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The complete document including Reference Table(s) and the Sequence Listing(s) can be downloaded from the EPO website

Description

1. INTRODUCTION

[0001] Described herein are chimeric influenza virus gene segments and nucleic acid sequences encoding such chimeric influenza virus gene segments. A chimeric influenza virus gene segment described herein comprises packaging signals found in the non-coding and coding regions of one type of influenza virus gene segment and an open reading frame of a different type of influenza virus gene segment or fragment thereof. Also described herein are recombinant influenza viruses comprising two or more chimeric influenza virus gene segments and the use of such viruses in the prevention and/or treatment of influenza virus disease.

2. BACKGROUND

[0002] Influenza viruses are enveloped RNA viruses that belong to the family of Orthomyxoviridae (Palese and Shaw (2007) Orthomyxoviridae: The Viruses and Their Replication, 5th ed. Fields' Virology, edited by B.N. Fields, D.M. Knipe and P.M. Howley. Wolters Kluwer Health/Lippincott Williams & Wilkins, Philadelphia, USA, p1647-1689). The natural host of influenza viruses are avians, but influenza viruses (including those of avian origin) also can infect and cause illness in humans and other animal hosts (canines, pigs, horses, sea mammals, and mustelids). For example, the HSN1 avian influenza virus circulating in Asia has been found in pigs in China and Indonesia and has also expanded its host range to include cats, leopards, and tigers, which generally have not been considered susceptible to influenza A (CIDRAP - Avian influenza: Agricultural and Wildlife Considerations). The occurrence of influenza virus infections in animals could potentially give rise to human pandemic influenza strains.

[0003] Influenza A and B viruses are major human pathogens, causing a respiratory disease that ranges in severity from sub-clinical infection to primary viral pneumonia which can result in death. The clinical effects of infection vary with the virulence of the influenza strain and the exposure, history, age, and immune status of the host. The cumulative morbidity and mortality caused by seasonal influenza is substantial due to the relatively high rate of infection. In a normal season, influenza can cause between 3-5 million cases of severe illness and up to 500,000 deaths worldwide (World Health Organization (2003) Influenza: Overview; who.int/mediacentre/factsheets/fs211/en/ website; March 2003). In the United States, influenza viruses infect an estimated 10-15% of the population (Glezen and Couch RB (1978) Interpandemic Influenza in the Houston area, 1974-76. N Engl J Med 298: 587-592; Fox et al. (1982) Influenza virus infections in Seattle families, 1975-1979. II. Pattern of infection in invaded households and relation of age and prior antibody to occurrence of infection and related illness. Am J Epidemiol 116: 228-242) and are associated with approximately 30,000 deaths each year (Thompson WW et al. (2003) Mortality Associated With Influenza and Respiratory Syncytial Virus in the United States. JAMA 289: 179-186; Belshe (2007) Translational research on vaccines: Influenza as an example. Clin Pharmacol Ther 82: 745-749).

[0004] In addition to annual epidemics, influenza viruses are the cause of infrequent pandemics. For example, influenza A viruses can cause pandemics such as those that occurred in 1918, 1957 and 1968. Due to the lack of pre-formed immunity against the major viral antigen, hemagglutinin (HA), pandemic influenza viruses can affect greater than 50% of the population in a single year and often cause more severe disease than seasonal influenza viruses. A stark example is the pandemic of 1918, in which an estimated 50-100 million people were killed (Johnson and Mueller (2002) Updating the Accounts: Global Mortality of the 1918-1920 "Spanish" Influenza Pandemic Bulletin of the History of Medicine 76: 105-115). Since the emergence of the highly pathogenic avian H5N1 influenza virus in the late 1990s (Claas et al. (1998) Human Influenza A H5N1 virus related to a highly pathogenic avian Influenza virus. Lancet 351: 472-7), there have been concerns that the virus may become transmissible between humans and cause a major pandemic. Recently, the World Health Organization has declared the H1N1 2009 swine influenza virus a pandemic virus.

[0005] Muramoto Y et al: "Hierarchy among viral RNA (vRNA) segments in their role in vRNA incorporation into influenza A virions", JOURNAL OF VIROLOGY MAR 2006 vol. 80, no. 5, March 2006 (2006-03), pages 2318-2325, relates to studies undertaken to understand the regions of the PB2, PB1, and PA viral RNAs of influenza virus A/WSN/33 that are important for the incorporation of the viral RNAs into virions. To assess these regions, Muramoto *et al.* discloses the generation of a series of mutant viral RNAs possessing the green fluorescent protein flanked by portions of the coding and non-coding regions of the PB2, PB1, and PA viral RNAs of influenza virus A/WSN/33.

[0006] Gao Q et al: "A seven-segmented influenza A virus expressing the influenza C virus glycoprotein HEF", JOURNAL OF VIROLOGY JUL 2008 vol. 82, no. 13, July 2008 (2008-07), pages 6419-6426 discloses the generation of a seven-segmented influenza A virus containing six RNA segments from influenza virus A/Puerto Rico/8/34 and an RNA segment containing the open reading frame (ORF) of influenza virus C/Johannesburg/1/66 glycoprotein HEF or the ORF of a chimeric protein HEF-Ectodomain flanked by packaging sequences of influenza virus A/WSN/33 HA. Gao *et al.* also discloses the generation of an eight-segmented influenza A virus with the seven segments and an eighth segment with the GFP ORF flanked by NA packaging sequences.

[0007] Fujii K et al: "Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions", JOURNAL OF VIROLOGY MAR 2005 vol. 79, no. 6, March 2005 (2005-03), pages 3766-3774, relates to studies undertaken to understand the regions of the NS viral RNA that are important for the incorporation of the NS viral RNA into virion. Fujii et al. identifies coding region and non-coding regions of the NS viral RNA that are required for efficient influenza A virus packaging.

[0008] An effective way to protect against influenza virus infection is through vaccination with attenuated influenza virus. However, due to reassortment, co-infection of an individual with a live attenuated vaccine strain and a wild-type strain of influenza could allow the formation of replication-competent virus carrying, *e.g.*, the vaccine-derived hemagglutinin, to which the infected person would likely to be naive. Accordingly, there is a need to develop methods of preventing the reassortment of vaccine strains of influenza virus with wild-type influenza viruses.

3. SUMMARY

[0009] Described herein are chimeric influenza virus gene segments and nucleic acid sequences encoding such chimeric influenza virus gene segments or the complement thereof which are useful in the production of recombinant influenza viruses. Two or more chimeric influenza virus gene segments or complements thereof, or nucleic acid sequences encoding such gene segments or the complements thereof may be used in the production of recombinant influenza viruses. Without being bound by any theory, the two or more chimeric influenza virus gene segments segregate together (*i.e.*, cosegregate) during replication of the recombinant influenza viruses such that the recombinant influenza viruses have a reduced ability to reassort with other influenza viruses (*e.g.*, wild-type influenza viruses) or are unable to reassort with other influenza viruses as determined by techniques known to one skilled in the art. The reduced ability or inability of such recombinant influenza viruses to reassort with other influenza viruses may improve the safety of the recombinant influenza viruses as a live attenuated vaccine. Accordingly, such recombinant influenza viruses may be useful in either the prevention of influenza virus disease, the treatment of influenza virus disease or influenza virus infection, or both.

[0010] The invention, as defined in the claims, provides a recombinant influenza A, B, or C virus, comprising influenza virus gene segments, wherein at least two of the influenza virus gene segments are chimeric influenza virus gene segments, wherein:

(a) a first chimeric influenza virus gene segment comprises, in the order presented:

(i) packaging signals found in the 3' non-coding region of a first influenza virus gene segment;
(ii) packaging signals found in the 3' proximal coding region of the first influenza virus gene segment, wherein the 3' proximal coding region of the first influenza virus gene segment has been mutated to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence;
(iii) an open reading frame from a second influenza virus gene segment, wherein the open reading frame contains one, two, three or more silent mutations in the virus packaging signals found in the 3' and 5' proximal nucleotides in the open reading frame;
(iv) packaging signals found in the 5' proximal coding region of the first influenza virus gene segment; and
(v) packaging signals found in the 5' non-coding region of the first influenza virus gene segment; and

(b) a second chimeric influenza virus gene segment comprises, in the order presented:

(i) packaging signals found in the 3' non-coding region of the second influenza virus gene segment;
(ii) packaging signals found in the 3' proximal coding region of the second influenza virus gene segment, wherein the 3' proximal coding region of the second influenza virus gene segment has been mutated to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence;
(iii) an open reading frame from the first influenza virus gene segment, wherein the open reading frame contains one, two, three or more silent mutations in the virus packaging signals found in the 3' and 5' proximal nucleotides in the open reading frame;
(iv) packaging signals found in the 5' proximal coding region of the second influenza virus gene segment; and
(v) packaging signals found in the 5' non-coding region of the second influenza virus gene segment;

wherein the first and second gene segments are two different gene segments, and wherein the recombinant influenza virus has a reduced ability to reassort with a different influenza virus such that the progeny influenza virus with the combined gene segments has reduced replication competence, wherein a virus with reduced replication competence is a virus that produces at least 1 log, 1.5 logs, 2 logs, 25 logs, 3 logs, 3.5 logs, 4 logs, 4.5 logs, 5 logs, 5.5 logs, 6 logs, 6.5 logs, 7 logs, 7.5 logs, 8 logs, 8.5 logs, 9 logs or 10 logs lower titers of replicating progeny than the replicating progeny produced by a wild-type influenza virus of the same type.

[0011] The invention further provides a recombinant influenza A, B, or C virus, comprising influenza virus gene segments, wherein at least three of the influenza virus gene segments are chimeric influenza virus gene segments, wherein:

(a) a first chimeric influenza virus gene segment comprises, in the order presented:

(i) packaging signals found in the 3' non-coding region of a third influenza virus gene segment;
(ii) packaging signals found in the 3' proximal coding region of the third influenza virus gene segment, wherein the 3' proximal coding region of the third influenza virus gene segment has been mutated to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence;
(iii) an open reading frame from a first influenza virus gene segment, wherein the open reading frame contains one, two, three or more silent mutations in the virus packaging signals found in the 3' and 5' proximal nucleotides in the open reading frame;
(iv) packaging signals found in the 5' proximal coding region of the third influenza virus gene segment; and
(v) packaging signals found in the 5' non-coding region of the third influenza virus gene segment; and

(b) a second chimeric influenza virus gene segment comprises, in the order presented:

(i) packaging signals found in the 3' non-coding region of the first influenza virus gene segment;
(ii) packaging signals found in the 3' proximal coding region of the first influenza virus gene segment, wherein the 3' proximal coding region of the first influenza virus gene segment has been mutated to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence;
(iii) an open reading frame from a second influenza virus gene segment, wherein the open reading frame contains one, two, three or more silent mutations in the virus packaging signals found in the 3' and 5' proximal nucleotides in the open reading frame;
(iv) packaging signals found in the 5' proximal coding region of the first influenza virus gene segment; and
(v) packaging signals found in the 5' non-coding region of the first influenza virus gene segment; and

(c) a third chimeric influenza virus gene segment comprising, in the order presented:

(i) packaging signals found in the 3' non-coding region of the second influenza virus gene segment;
(ii) packaging signals found in the 3' proximal coding region of the second influenza virus gene segment, wherein the 3' proximal coding region of the second influenza virus gene segment has been mutated to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence;
(iii) an open reading frame from the third influenza virus gene segment, wherein the open reading frame contains one, two, three or more silent mutations in the virus packaging signals found in the 3' and 5' proximal nucleotides in the open reading frame;
(iv) packaging signals found in the 5' proximal coding region of the second influenza virus gene segment; and
(v) packaging signals found in the 5' non-coding region of the second influenza virus gene segment;

wherein the first, second and third gene segments are three different gene segments, and
wherein the recombinant influenza virus has a reduced ability to reassort with a different influenza virus such that the progeny influenza virus with the combined gene segments has reduced replication competence, wherein a virus with reduced replication competence is a virus that produces at least 1 log, 1.5 logs, 2 logs, 25 logs, 3 logs, 3.5 logs, 4 logs, 4.5 logs, 5 logs, 5.5 logs, 6 logs, 6.5 logs, 7 logs, 7.5 logs, 8 logs, 8.5 logs, 9 logs or 10 logs lower titers of replicating progeny than the replicating progeny produced by a wild-type influenza virus of the same type.

[0012] The embodiments of recombinant viruses disclosed in the subsequent paragraphs of this description comprise the features of the recombinant viruses of the invention described above. The embodiments of gene segments and nucleic acids disclosed in the subsequent paragraphs of this description are to be interpreted as gene segments and nucleic acids comprising/encoding same of the recombinant viruses of the invention or used in the production of the recombinant viruses of the invention.

[0013] In some embodiments, the first, second or third influenza virus gene segment refers to an HA, NA, NS, PB1, PB2, PA, M, or NP gene segment from an influenza virus.

[0014] In some embodiments, the first chimeric influenza virus gene segment encodes HA protein and the second chimeric influenza virus gene segment encodes NA protein. In one embodiment, the second chimeric influenza virus segment comprises:

(a) the 3' non-coding region nucleotide sequence consisting of SEQ ID NO: 19 and the 3' proximal coding region

sequence consisting of SEQ ID NO: 20, and
(b) the 5' non-coding region nucleotide sequence consisting of SEQ ID NO: 22 and the 5' proximal coding region sequence consisting of SEQ ID NO: 23.

[0015] In another embodiment, the first chimeric influenza virus gene segment comprises:

(a) the 3' non-coding region nucleotide sequence consisting of SEQ ID NO: 31 and the 3' proximal coding region sequence consisting of SEQ ID NO: 32, and
(b) the 5' non-coding region nucleotide sequence consisting of SEQ ID NO: 34 and a 5' proximal coding region sequence consisting of SEQ ID NO: 35.

[0016] In some embodiments, the first, second, and third influenza virus gene segments encode the HA, NA, and NS proteins, respectively.

[0017] In some embodiments, the first chimeric influenza virus gene segment encodes NS protein, and wherein the 3' proximal coding region is mutated so as to eliminate the mRNA 5' splice site.

[0018] In some embodiments, the first chimeric influenza virus gene segment encodes M protein, and wherein the 3' proximal coding region is mutated so as to eliminate the mRNA 5' splice site.

[0019] In some embodiments, the recombinant influenza virus reassorts with other influenza viruses by less than 5%, as determined by the percentage of viral plaques containing reassorted influenza viruses with one or more chimeric influenza virus gene segments that have reassorted independently from one or more other chimeric influenza virus gene segments.

[0020] In some embodiments, the virus is attenuated.

[0021] The invention further provides a host comprising the recombinant influenza virus of the invention, wherein the host is a chicken or avian cell, a cell from a cell line, or a chicken or avian embryonated egg.

[0022] The invention further provides a pharmaceutical composition or an immunogenic composition comprising the recombinant influenza virus of the invention.

[0023] The invention further provides the recombinant influenza virus of the invention, for use in eliciting an immune response against an influenza virus in a subject; for use in preventing or treating an influenza virus disease in a subject; and for use in treating an influenza virus infection in a subject.

[0024] The invention further provides the composition of the invention, for use in eliciting an immune response against an influenza virus in a subject; for use in preventing or treating an influenza virus disease in a subject; and for use in treating an influenza virus infection in a subj ect.

[0025] In some embodiments, wherein the subject is a human.

[0026] The references to methods of treatment in the subsequent paragraphs of this description are to be interpreted as references to the compounds, pharmaceutical compositions and medicaments of the present invention for use in a method for treatment of the human (or animal) body by therapy.

[0027] The invention provides a method for propagating a recombinant influenza virus, wherein the method comprises infecting a chicken or avian cell or a cell from a cell line with the recombinant influenza virus of the invention; and purifying the virus subsequently from said cell.

[0028] The invention further provides a method for propagating a recombinant influenza virus, wherein the method comprises infecting a chicken or avian embryonated egg with the recombinant influenza virus of the invention; and purifying the virus subsequently from said egg.

[0029] A chimeric influenza virus gene segment of the recombinant virus comprises: (a) packaging signals found in the 3' and the 5' non-coding regions of a first type of influenza virus gene segment, (b) packaging signals found in the 3' proximal coding region sequence of the first type of influenza virus gene segment, and the 5' proximal coding region sequence of the first type of influenza virus gene segment, and (c) an open reading frame or a fragment thereof from a second, different type of influenza virus gene segment, wherein the open reading frame contains one, two, three or more mutations in the influenza virus packaging signals found in the open reading frame. The 3' and the 5' proximal coding regions sequences flank the open reading frame. In certain embodiments, the 3' and the 5' proximal coding region sequences flank the open reading frame and are not translated. The 3' proximal coding region sequence has been mutated so as to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence. In certain embodiments, the 3' proximal coding region is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3' proximal coding region is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated to eliminate the mRNA 5' splice site. In another specific embodiment, the 3' proximal coding region is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated to eliminate the distal 5' splice site. In some embodiments, the 5' proximal coding region sequence has one or more mutations so as to ensure that the 5' proximal coding region sequence is not translated. The mutations introduced into the open reading frame of the influenza virus gene segment or a fragment are silent mutations.

[0030] In one embodiment, a chimeric influenza virus gene segment of the recombinant virus comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) at least the 3' proximal 20 nucleotides from an open reading frame of a second type of influenza virus gene segment, wherein the at least 20 nucleotides carry one or more mutations; (iv) a 5' proximal coding region of the first type of influenza virus gene segment; and (v) the 5' non-coding region of the first type of influenza virus gene segment, wherein the nucleic acid is engineered such that an open reading frame may be inserted following in frame with the at least 20 nucleotides of the open reading frame of the second type of influenza virus gene segment. In another embodiment, a chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) at least the 5' proximal 30 nucleotides of an open reading frame of a second type of influenza virus gene segment, wherein the at least 30 nucleotides carry one or more mutations; (iv) a 5' proximal coding region of the first type of influenza virus gene segment; and (v) the 5' non-coding region of the first type of influenza virus gene segment, wherein the nucleic acid is engineered such that an open reading frame may be inserted preceding in frame with the at least 30 nucleotides of the open reading frame of the second type of influenza virus gene segment. In another embodiment, a chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) at least the 3' proximal 20 nucleotides of an open reading frame of a second type of influenza virus gene segment, wherein the at least 20 nucleotides carry one or more mutations; (iv) at least the 5' proximal 30 nucleotides of the open reading frame of the second type of influenza virus gene segment, wherein the at least 30 nucleotides carry one or more mutations; (v) a 5' proximal coding region of the first type of influenza virus gene segment; and (vi) the 5' non-coding region of the first type of influenza virus gene segment, wherein the nucleic acid is engineered such that an open reading frame may be inserted in frame between the at least 20 nucleotides from the open reading frame of the second type of influenza virus gene segment and the at least 30 nucleotides of the open reading frame of the second type of influenza virus gene segment. In certain embodiments, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site.

[0031] In a specific embodiment, a chimeric influenza virus gene segment of the recombinant virus comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) an open reading frame of a second type of influenza virus gene segment, wherein the 3' proximal nucleotides and the 5' proximal nucleotides of the open reading frame are mutated; (iv) a 5' proximal coding region of the first type of influenza virus gene segment; and (v) the 5' non-coding region of the first type of influenza virus gene segment.

[0032] Described herein are nucleic acid sequences comprising the complement of a chimeric influenza virus gene segment which may be useful in the production of recombinant influenza viruses. In specific cases, a nucleic acid sequence comprises the complement of a chimeric influenza virus gene segment, wherein the chimeric influenza virus gene segment comprises: (a) packaging signals found in the 3' and the 5' non-coding regions of a first type of influenza virus gene segment, (b) packaging signals found in the 3' proximal coding region sequence of the first type of influenza virus gene segment, the 5' proximal coding region sequence of the first type of influenza virus gene segment, or both the 3' and the 5' proximal coding region sequences of the first type of influenza virus gene segment, and (c) an open reading frame or a fragment thereof from a second, different type of influenza virus gene segment, wherein the open reading frame contains one, two, three or more mutations in the influenza virus packaging signals found in the open reading frame. In certain cases, the 3' and/or the 5' proximal coding regions sequences flank the open reading frame and are translated in frame with the open reading frame. In other cases, the 3' and/or the 5' proximal coding region sequences flank the open reading frame and are not translated. In some cases, the 3' proximal coding region sequence has been mutated so as to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence. In certain cases, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific case, the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific case, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site. In some cases, the 5' proximal coding region sequence has one or more mutations so as to ensure that the 5' proximal coding region sequence is not translated. In a specific case, the mutations introduced into the open reading frame of the influenza virus gene segment or a fragment are silent mutations.

[0033] In one case, a nucleic acid sequence described herein comprises the complement of a chimeric influenza virus gene segment, wherein the chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) at least the 3' proximal 20 nucleotides from an open reading frame of a second type of influenza virus gene segment, wherein the at least 20 nucleotides carry one or more mutations; (iv) a 5' proximal coding region of the first type of influenza virus gene segment; and (v) the 5' non-coding region of the first type of influenza virus gene segment, wherein the nucleic acid is engineered such that an open reading frame may be inserted following in frame with the at least 20 nucleotides of the open reading frame of the second type of influenza virus gene segment. In anothercase, a nucleic acid sequence provided herein comprises the complement of a chimeric influenza virus gene segment, wherein the chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) at least the 5' proximal 30 nucleotides of an open reading frame of a second type of influenza virus gene segment, wherein the at least 30 nucleotides carry one or more mutations; (iv) a 5' proximal coding region of the first type of influenza virus gene segment; and (v) the 5' non-coding region of the first type of influenza virus gene segment, wherein the nucleic acid is engineered such that an open reading frame may be inserted preceding in frame with the at least 30 nucleotides of the open reading frame of the second type of influenza virus gene segment. In another case, a nucleic acid sequence provided herein comprises the complement of a chimeric influenza virus gene segment, wherein the chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) at least the 3' proximal 20 nucleotides of an open reading frame of a second type of influenza virus gene segment, wherein the at least 20 nucleotides carry one or more mutations; (iv) at least the 5' proximal 30 nucleotides of the open reading frame of the second type of influenza virus gene segment, wherein the at least 30 nucleotides carry one or more mutations; (v) a 5' proximal coding region of the first type of influenza virus gene segment; and (vi) the 5' non-coding region of the first type of influenza virus gene segment, wherein the nucleic acid is engineered such that an open reading frame may be inserted in frame between the at least 20 nucleotides from the open reading frame of the second type of influenza virus gene segment and the at least 30 nucleotides of the open reading frame of the second type of influenza virus gene segment. In certain cases, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific case, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific case, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site.

[0034] In a specific case, a nucleic acid sequence described herein comprises the complement of a chimeric influenza virus gene segment, wherein the chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) an open reading frame of a second type of influenza virus gene segment, wherein the 3' proximal nucleotides and the 5' proximal nucleotides of the open reading frame are mutated; (iv) a 5' proximal coding region of the first type of influenza virus gene segment; and (v) the 5' non-coding region of the first type of influenza virus gene segment. In certain embodiments, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific case, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific case, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site.

[0035] Described herein are nucleic acid sequences comprising a nucleotide sequence encoding a chimeric influenza virus gene segment or the complement thereof which may be useful in the production of recombinant influenza viruses. In specific cases, a nucleic acid sequence provided herein comprises a nucleotide sequence encoding a chimeric influenza virus gene segment or the complement thereof, wherein the chimeric influenza virus gene segment comprises: (a) packaging signals found in the 3' and the 5' non-coding regions of a first type of influenza virus gene segment, (b) packaging signals found in the 3' proximal coding region sequence of the first type of influenza virus gene segment, the 5' proximal coding region sequence of the first type of influenza virus gene segment, or both the 3' and the 5' proximal coding region sequences of the first type of influenza virus gene segment, and (c) an open reading frame or a fragment thereof from a second, different type of influenza virus gene segment, wherein the open reading frame contains one, two, three or more mutations in the influenza virus packaging signals found in the open reading frame. In certain cases, the 3' and/or the 5' proximal coding regions sequences flank the open reading frame and are translated in frame with the open reading frame. In other cases, the 3' and/or the 5' proximal coding region sequences flank the open reading frame and are not translated. In some cases, the 3' proximal coding region sequence has been mutated so as to eliminate

any start codons and preclude the translation of the 3' proximal coding region sequence. In certain cases, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific case, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site. In some cases, the 5' proximal coding region sequence has one or more mutations so as to ensure that the 5' proximal coding region sequence is not translated. In a specific case, the mutations introduced into the open reading frame of the influenza virus gene segment or a fragment are silent mutations.

[0036] In one case, a nucleic acid sequence described herein comprises a nucleotide sequence encoding a chimeric influenza virus gene segment or the complement thereof, wherein the chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) at least the 3' proximal 20 nucleotides from an open reading frame of a second type of influenza virus gene segment, wherein the at least 20 nucleotides carry one or more mutations; (iv) a 5' proximal coding region of the first type of influenza virus gene segment; and (v) the 5' non-coding region of the first type of influenza virus gene segment, wherein the nucleic acid is engineered such that an open reading frame may be inserted following in frame with the at least 20 nucleotides of the open reading frame of the second type of influenza virus gene segment. In another case, a nucleic acid sequence described herein comprises a nucleotide sequence encoding a chimeric influenza virus gene segment or the complement thereof, wherein the chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) at least the 5' proximal 30 nucleotides of an open reading frame of a second type of influenza virus gene segment, wherein the at least 30 nucleotides carry one or more mutations; (iv) a 5' proximal coding region of the first type of influenza virus gene segment; and (v) the 5' non-coding region of the first type of influenza virus gene segment, wherein the nucleic acid is engineered such that an open reading frame may be inserted preceding in frame with the at least 30 nucleotides of the open reading frame of the second type of influenza virus gene segment. In another case, a nucleic acid sequence described herein comprises a nucleotide sequence encoding a chimeric influenza virus gene segment or the complement thereof, wherein the chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) at least the 3' proximal 20 nucleotides of an open reading frame of a second type of influenza virus gene segment, wherein the at least 20 nucleotides carry one or more mutations; (iv) at least the 5' proximal 30 nucleotides of the open reading frame of the second type of influenza virus gene segment, wherein the at least 30 nucleotides carry one or more mutations; (v) a 5' proximal coding region of the first type of influenza virus gene segment; and (vi) the 5' non-coding region of the first type of influenza virus gene segment, wherein the nucleic acid is engineered such that an open reading frame may be inserted in frame between the at least 20 nucleotides from the open reading frame of the second type of influenza virus gene segment and the at least 30 nucleotides of the open reading frame of the second type of influenza virus gene segment. In certain cases, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific case, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific case, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site.

[0037] In a specific case, a nucleic acid sequence described herein comprises a nucleotide sequence encoding a chimeric influenza virus gene segment or the complement thereof, wherein the chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) an open reading frame of a second type of influenza virus gene segment, wherein the 3' proximal nucleotides and the 5' proximal nucleotides of the open reading frame are mutated; (iv) a 5' proximal coding region of the first type of influenza virus gene segment; and (v) the 5' non-coding region of the first type of influenza virus gene segment. In certain cases, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific case, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific case, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site.

[0038] In another aspect, provided herein are recombinant influenza viruses comprising two, three, four, five, six, seven or eight chimeric influenza virus gene segments described herein. In a specific embodiment, provided herein are recombinant influenza viruses comprising two or more chimeric influenza virus gene segments described herein, wherein

the two or more chimeric influenza virus gene segments cosegregate. Without being bound by theory, the chimeric influenza virus gene segments have a reduced ability to reassort independently of each other with other influenza virus gene segments, and thus, the reassortment of the recombinant influenza virus with other influenza viruses (e.g., wild-type influenza viruses) is reduced or inhibited. Recombinant influenza viruses that are unable to reassort will produce fewer viral plaques that contain viruses with one or more chimeric influenza virus gene segments that has reassorted independently of one or more other chimeric influenza virus gene segments. In certain embodiments, a recombinant influenza virus described herein comprises an attenuating mutation.

[0039] In one embodiment, a recombinant influenza virus comprising a first chimeric influenza virus gene segment and a second chimeric influenza virus gene segment, wherein (a) the first chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) the open reading frame of a second type of influenza virus gene segment, wherein 3' and the 5' proximal nucleotides in the open reading frame are mutated; (iv) a 5' proximal coding region of the first type of influenza virus gene segment; and (v) the 5' non-coding region of the first type of influenza virus gene segment; and wherein (b) the second chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of a second type of influenza virus gene segment; (ii) a 3' proximal coding region of the second type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the second type of influenza virus gene segment is mutated; (iii) the open reading frame of a first type of influenza virus gene segment, wherein 3' and the 5' proximal nucleotides in the open reading frame are mutated; (iv) a 5' proximal coding region of the second type of influenza virus gene segment; and (v) the 5' non-coding region of the second type of influenza virus influenza gene segment.

[0040] In another embodiment, a recombinant influenza virus comprises a first chimeric influenza virus gene segment; a second chimeric influenza virus gene segment; and a third chimeric influenza virus gene segment, wherein (a) the first chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of a third type of influenza virus gene segment; (ii) a 3' proximal coding region of the third type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the third influenza virus gene segment is mutated; (iii) the open reading frame of a first type of influenza virus gene segment, wherein 3' and the 5' proximal nucleotides in the open reading frame are mutated; (iv) a 5' proximal coding region of the third type of influenza virus gene segment; and (v) the 5' non-coding region of the third type of influenza virus gene segment; and wherein (b) the second chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of the first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) the open reading frame of a second type of influenza virus gene segment, wherein 3' and the 5' proximal nucleotides in the open reading frame are mutated; (iv) a 5' proximal coding region of the first type of influenza virus gene segment; and (v) the 5' non-coding region of the first type of influenza virus gene segment; and wherein (c) the third chimeric influenza virus gene segment comprises: (i) the 3' non-coding region of the second type of influenza virus gene segment; (ii) a 3' proximal coding region of the second type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the second type of influenza virus gene segment is mutated; (iii) the open reading frame of the third type of influenza virus gene segment, wherein 3' and the 5' proximal nucleotides in the open reading frame are mutated; (iv) a 5' proximal coding region of the second type of influenza virus gene segment; and (v) the 5' non-coding region of the second type of influenza virus gene segment. In certain embodiments, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site.

[0041] In another aspect, provided herein are recombinant influenza viruses containing nine gene segments, wherein at least two of the gene segments are chimeric influenza virus gene segments such as described herein. In certain embodiments, a recombinant influenza virus comprises nine gene segments, wherein (a) at least one gene segment comprises: (i) the packaging signals found in the 3' non-coding region of a first type of influenza virus gene segment or a derivative thereof; (ii) the packaging signals found in the 3' proximal coding region of the first type of influenza virus gene segment or a derivative thereof, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) an open reading frame of a second type of influenza virus gene segment or a fragment or a derivative thereof, wherein the 3' and 5' proximal nucleotides in the open reading frame are mutated; (iv) the packaging signals found in the 5' proximal coding region of the first type of influenza virus gene segment or a derivative thereof; and (v) the packaging signals found in the 5' non-coding region of the first type of influenza virus gene segment or a derivative thereof; and (b) at least one gene segment comprises: (i) the packaging signals found in the 3' non-coding region of the second type of influenza virus gene segment or a derivative thereof; (ii) the packaging signals found in the 3' proximal coding region of the second type of influenza virus gene segment or a derivative thereof, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) an open reading

frame heterologous to 1, 2, 3, 4, 5, 6, 7 or 8 of the influenza virus gene segment; (iv) the packaging signals found in the 5' proximal coding region of the second type of influenza virus gene segment or a derivative thereof; and (v) the packaging signals found in the 5' non-coding region of the second type of influenza virus gene segment or a derivative thereof. In certain embodiments, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site. In certain embodiments, the recombinant influenza virus is attenuated. In some embodiments, the recombinant influenza virus encodes and/or expresses influenza virus antigens from two different types, subtypes or strains of influenza virus. In a specific embodiment, the recombinant influenza virus encodes and/or expresses HA antigens from two different types, subtypes or strains of influenza virus. For example the recombinant influenza virus encodes and/or expresses an H1 HA and an H3 HA antigen. In some embodiments, the one HA antigens is from a seasonal influenza virus and the other HA antigen is from a pandemic influenza virus. In specific embodiments, each of the two HA antigens comprise an attenuating mutation. In certain embodiments, the recombinant influenza virus encodes and/or expresses influenza virus antigens and at least one, two, three or four, or 1 to 3, 1 to 4, or 2 to 4 non-influenza virus antigens (*e.g.,* antigens from bacterial pathogens, or viral pathogens other an influenza virus). In accordance with these embodiments, in some embodiments, the heterologous open reading frame of the one gene segment can encode an influenza virus antigen from a different type, subtype or strain of influenza virus than the influenza virus antigens encoded by the other gene segments. In other embodiments, the heterologous open reading frame of the one gene segment can encode a non-influenza virus antigen (*e.g.,* a bacterial antigen, tumor antigen, or viral antigen other than an influenza virus antigen).

[0042] In specific embodiments, the nine-segmented recombinant influenza viruses described herein more stably incorporate the ninth segment than those previously described. In certain embodiments, the nine-segmented recombinant influenza viruses described herein maintain the ninth segment over at least 4, 5, 6, 7, 8 or more passages, or 4 to 6, 4 to 8, or 5 to 8 passages in embryonated eggs or tissue culture as assessed by techniques known in the art (including, *e.g.*, the limiting dilution technique described in the examples *infra*).

[0043] Described herein are substrates (*e.g.*, host cells and eggs) comprising a nucleic acid sequence described herein. In one case, described herein are substrates comprising a chimeric influenza virus gene segment or a complement thereof. In another case, described herein are substrates comprising a nucleic acid sequence comprising a nucleotide sequence encoding a chimeric influenza virus gene segment or a complement thereof.

[0044] In another aspect, provided herein are substrates comprising a recombinant influenza virus comprising two or more chimeric influenza virus gene segments described herein. In another aspect, provided herein are compositions comprising a recombinant influenza virus comprising two or more chimeric influenza virus gene segments described herein.

[0045] Described herein are kits comprising a nucleic acid sequence or recombinant influenza virus described herein. Described one case, a kit described herein comprises, in one or more containers, a nucleic acid sequence described herein. In another case, a kit described herein, comprises, in one or more containers, a recombinant influenza virus described herein.

[0046] In yet another aspect, provided herein are methods of using a recombinant influenza virus comprising one, two or more chimeric influenza virus gene segments. In one embodiment, provided herein is a method for eliciting an immune response against an influenza virus in a subject, wherein the method comprises administering a recombinant influenza virus described herein or a composition thereof to the subject. In another embodiment, provided herein is a method of preventing and/or treating an influenza virus infection in a subject, wherein the method comprises administering a recombinant influenza virus described herein or a composition thereof to the subject. In another embodiment, provided herein is a method for preventing and/or treating an influenza virus disease in a subject, wherein the method comprises administering a recombinant influenza virus described herein or a composition thereof to the subject. Described herein are methods for generating or identifying antibodies that bind to an influenza virus utilizing a recombinant influenza virus described herein or a composition thereof.

3.1 TERMINOLOGY

[0047] As used herein, the phrase "ability to reassort" in the context of an influenza virus gene segment or a chimeric influenza virus gene segment is used to describe the ability of the influenza virus gene segment or the chimeric influenza virus gene segment to segregate independently from other influenza virus gene segments or chimeric influenza virus gene segments through at least one life cycle of the influenza virus and to encode a replication competent virus in combination with the remainder of the influenza virus gene segments in an influenza virus genome. In the context of an influenza virus, the phrase "ability to reassort" is used herein to describe the ability of the influenza virus to combine any one of its gene segments with the gene segments of a different influenza virus such that the progeny influenza virus

with the combined gene segments is replication competent. An influenza virus has a reduced ability to reassort if certain combinations of the mixed gene segments do not yield replication competent virus or a virus with reduced replication competence. In certain embodiments, an influenza virus with reduced replication competence is a virus that produces at least 1 log, 1.5 logs, 2 logs, 25 logs, 3 logs, 3.5 logs, 4 logs, 4.5 logs, 5 logs, 5.5 logs, 6 logs, 6.5 logs, 7 logs, 7.5 logs, 8 logs, 8.5 logs, 9 logs or 10 logs lower titers of replicating progeny than the replicating progeny produced by a wild-type influenza virus of the same type.

[0048] As used herein, the term "about" or "approximately" when used in conjunction with a number refers to any number within 1, 5 or 10% of the referenced number.

[0049] As used herein, the term "derivative" in the context of an influenza virus gene segment refers to a nucleotide sequence that is at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98% identical to a particular nucleotide sequence of an influenza virus, or a nucleotide sequence that hybridizes under stringent conditions to a particular nucleotide sequence of an influenza virus.

[0050] As used herein, the term "effective amount" in the context of administering a therapy to a subject refers to the amount of a therapy which has a prophylactic and/or therapeutic effect(s). In certain embodiments, an "effective amount" in the context of administration of a therapy to a subject refers to the amount of a therapy which is sufficient to achieve one, two, three, four, or more of the following effects: (i) reduction or amelioration in the severity of an influenza virus infection, an influenza virus disease or symptom associated therewith; (ii) reduction in the duration of an influenza virus infection, an influenza virus disease or symptom associated therewith; (iii) prevention of the progression of an influenza virus infection, an influenza virus disease or symptom associated therewith; (iv) regression of an influenza virus infection, an influenza virus disease or symptom associated therewith; (v) prevention of the development or onset of an influenza virus infection, an influenza virus disease or symptom associated therewith; (vi) prevention of the recurrence of an influenza virus infection, an influenza virus disease or symptom associated therewith; (vii) reduction or prevention of the spread of an influenza virus from one cell to another cell, one tissue to another tissue, or one organ to another organ; (viii) prevention or reduction of the spread/transmission of an influenza virus from one subject to another subject; (ix) reduction in organ failure associated with an influenza virus infection or influenza virus disease; (x) reduction in the hospitalization of a subject; (xi) reduction in the hospitalization length; (xii) an increase in the survival of a subject with an influenza virus infection or a disease associated therewith; (xiii) elimination of an influenza virus infection or a disease associated therewith; (xiv) inhibition or reduction in influenza virus replication; (xv) inhibition or reduction in the binding or fusion of influenza virus to a host cell(s); (xvi) inhibition or reduction in the entry of an influenza virus into a host cell(s); (xvii) inhibition or reduction of the replication of the influenza virus genome; (xviii) inhibition or reduction in the synthesis of influenza virus proteins; (xix) inhibition or reduction in the assembly of influenza virus particles; (xx) inhibition or reduction in the release of influenza virus particles from a host cell(s); (xxi) reduction in influenza virus titer; (xxii) reduction in the number of symptoms associated with an influenza virus infection or an influenza virus disease; (xxiii) enhancement, improvement, supplementation, complementation, or augmentation of the prophylactic or therapeutic effect(s) of another therapy; (xxiv) prevention of the onset or progression of a secondary infection associated with an influenza virus infection; and/or (xxv) prevention of the onset or diminution of disease severity of bacterial pneumonias occurring secondary to influenza virus infections. Exemplary doses of an effective amount are provided in Section 5.7.2, *infra.*

[0051] In certain embodiments, the effective amount of a therapy does not result in complete protection from an influenza virus disease, but results in a lower titer or reduced number of influenza viruses compared to an untreated subject. In certain embodiments, the effective amount of a therapy results in a 0.5 fold, 1 fold, 2 fold, 4 fold, 6 fold, 8 fold, 10 fold, 15 fold, 20 fold, 25 fold, 50 fold, 75 fold, 100 fold, 125 fold, 150 fold, 175 fold, 200 fold, 300 fold, 400 fold, 500 fold, 750 fold, or 1,000 fold or greater reduction in titer of Influenza virus relative to an untreated subject. In some embodiments, the effective amount of a therapy results in a reduction in titer of influenza virus relative to an untreated subject of approximately 1 log or more, approximately 2 logs or more, approximately 3 logs or more, approximately 4 logs or more, approximately 5 logs or more, approximately 6 logs or more, approximately 7 logs or more, approximately 8 logs or more, approximately 9 logs or more, approximately 10 logs or more, 1 to 5 logs, 2 to 10 logs, 2 to 5 logs, or 2 to 8 logs. Benefits of a reduction in the titer, number or total burden of influenza virus include, but are not limited to, less severe symptoms of the infection, fewer symptoms of the infection, reduction in the length of the disease associated with the infection, and prevention of the onset or diminution of disease severity of bacterial pneumonias occurring secondary to influenza virus infections.

[0052] As used herein, the term "elderly human" refers to a human 65 years or older.

[0053] As used herein, the term "fragment" in the context of a nucleic acid sequence refers to a nucleotide sequence comprising at least 2 or at least 3 consecutive nucleotides from a parent sequence. In a specific embodiment, the term refers to a nucleotide sequence of 2 to 30, 5 to 30, 10 to 60, 25 to 100, 150 to 300 or more consecutive nucleotides from a parent sequence. In another embodiment, the term refers to a nucleotide sequence of at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 125, 150, 175, 200, 250, 275, 300, 325, 350, 375, 400, 425, 450 or 475 consecutive nucleotides of a parent sequence.

[0054] As used herein, the term "fragment" in the context of an amino acid sequence refers to an amino acid sequence

comprising at least 2 consecutive amino acid residues from a parent sequence. In a specific embodiment, the term refers to an amino acid sequence of 2 to 30, 5 to 30, 10 to 60, 25 to 100, 150 to 300 or more consecutive amino acid residues from a parent sequence. In another embodiment, the term refers to an amino acid sequence of at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 110, 125, 150, 175, 200, 250, 275, 300, 325, 350, 375, 400, 425, 450 or 475 consecutive amino acid residues of a parent sequence.

[0055] As used herein, the term "heterologous" refers to a unit that is not found naturally be associated with another unit. For example, a first nucleotide sequence is said be a heterologous to a second nucleotide sequence if the two nucleotide sequences are not found in nature to be associated with each other.

[0056] As used herein, the term "host cell" refers to any type of cell, *e.g.,* a primary cell or a cell from a cell line. In specific embodiments, the term "host cell" refers a cell transfected with a nucleic acid molecule and the progeny or potential progeny of such a cell. Progeny of such a cell may not be identical to the parent cell transfected with the nucleic acid molecule due to mutations or environmental influences that may occur in succeeding generations or integration of the nucleic acid molecule into the host cell genome.

[0057] As used herein, the term "human adult" refers to a human that is 18 years or older.

[0058] As used herein, the term "human child" refers to a human that is 1 year to 18 years old.

[0059] As used herein, the term "human infant" refers to a newborn to 1 year old human.

[0060] As used herein, the term "hybridizes under stringent conditions" describes conditions for hybridization and washing under which nucleotide sequences at least 50% (preferably, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 98%) identical to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6.

[0061] Generally, stringent conditions are selected to be about 5 to 10°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength pH. The Tm is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at Tm, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (for example, 10 to 50 nucleotides) and at least 60°C for long probes (for example, greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents, for example, formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

[0062] In one, non-limiting example stringent hybridization conditions are hybridization at 6x sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.1x.SSC, 0.2% SDS at about 68°C. In a specific, non-limiting example stringent hybridization conditions are hybridization in 6xSSC at about 45°C, followed by one or more washes in 0.2xSSC, 0.1% SDS at 50-65°C (*i.e.,* one or more washes at 50°C, 55°C, 60°C or 65°C). It is understood that the nucleic acids described herein do not include nucleic acid molecules that hybridize under these conditions solely to a nucleotide sequence consisting of only A or T nucleotides.

[0063] As used herein, the term "in combination" in the context of the administration of a therapy(ies) to a subject, refers to the use of more than one therapy. The use of the term "in combination" does not restrict the order in which therapies are administered to a subject. A first therapy can be administered prior to (*e.g.,* 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks before), concomitantly with, or subsequent to (*e.g.,* 5 minutes, 15 minutes, 30 minutes, 45 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 8 weeks, or 12 weeks after) the administration of a second therapy to a subject.

[0064] As used herein, the term "infection" means the invasion by, multiplication and/or presence of a virus in a cell or a subject. In one embodiment, an infection is an "active" infection, *i.e.,* one in which the virus is replicating in a cell or a subject. Such an infection is characterized by the spread of the virus to other cells, tissues, and/or organs, from the cells, tissues, and/or organs initially infected by the virus. An infection may also be a latent infection, *i.e*., one in which the virus is not replicating. In certain embodiments, an infection refers to the pathological state resulting from the presence of the virus in a cell or a subject, or by the invasion of a cell or subject by the virus.

[0065] As used herein, the term "influenza virus disease" and phrases referring to a disease associated with an influenza virus infection refer to the pathological state resulting from the presence of an influenza virus (*e.g.,* influenza A or B virus) in a cell or subject or the invasion of a cell or subject by an influenza virus. In specific embodiments, the term refers to a respiratory illness caused by an influenza virus.

[0066] As used herein, the phrases "IFN-deficient systems" or "IFN-deficient substrates" refer to systems, *e.g*., cells, cell lines and animals, such as mice, chickens, turkeys, rabbits, rats, horses etc., which (a) do not produce one, two or more types of IFN, or do not produce any type of IFN, or produce low levels of one, two or more types of IFN, or produce low levels of any IFN (*i.e.,* a reduction in any IFN expression of 5-10%, 10-20%, 20-30%, 30-40%, 40-50%, 50-60%,

60-70%, 70-80%, 80-90% or more when compared to IFN-competent systems under the same conditions), (b) do not respond or respond less efficiently to one, two or more types of IFN, or do not respond to any type of IFN, and/or (c) are deficient in the activity of antiviral genes induced by one, two or more types of IFN, or induced by any type of IFN.

[0067] An "isolated" protein (*e.g.,* an antibody) is substantially free of cellular material or heterologous proteins (also referred to herein as contaminating proteins) from the cell or tissue source from which the protein is derived, or substantially free of chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of a protein (*e.g.,* an antibody) in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. Thus, a protein (*e.g.,* an antibody) that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of heterologous protein. When the protein is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.,* culture medium represents less than about 20%, 10%, or 5% of the volume of the protein preparation. When the protein is produced by chemical synthesis, it is preferably substantially free of chemical precursors or other chemicals, *i.e.,* it is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. Accordingly such preparations of the protein have less than about 30%, 20%, 10%, 5% (by dry weight) of chemical precursors or compounds other than the protein of interest. In another specific embodiment, antibodies described herein are isolated.

[0068] As used herein, the term "isolated" in the context of nucleic acids refers to a nucleic acid molecule which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid molecule or substantially free of chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized; however, "isolated" excludes members of a library of clones such as a cDNA library. In a specific embodiment, a nucleic acid described herein is isolated.

[0069] As used herein, the terms "manage," "managing," and "management" refer to the beneficial effects that a subject derives from a therapy (*e.g.,* a prophylactic or therapeutic agent), which does not result in a cure of the infection or disease associated therewith. In certain embodiments, a subject is administered one or more therapies (*e.g.,* prophylactic or therapeutic agents) to "manage" an influenza virus disease, or one or more symptoms thereof, so as to prevent the progression or worsening of the disease.

[0070] As used herein, the phrase "multiplicity of infection" or "MOI" is the average number of virus per infected cell. The MOI is determined by dividing the number of virus added (ml added x plaque forming units (pfu)) by the number of cells added (ml added x cells/ml).

[0071] As used herein, the term "nucleic acid" refers to deoxyribonucleotides, deoxyribonucleic acids, ribonucleotides, and ribonucleic acids, and polymeric forms thereof, and includes either single- or double-stranded forms. Nucleic acids include naturally occurring nucleic acids, such as deoxyribonucleic acid ("DNA") and ribonucleic acid ("RNA") as well as nucleic acid analogs. Nucleic acid analogs include those which include non-naturally occurring bases, nucleotides that engage in linkages with other nucleotides other than the naturally occurring phosphodiester bond or which include bases attached through linkages other than phosphodiester bonds. Thus, nucleic acid analogs include, for example and without limitation, phosphorothioates, phosphorodithioates, phosphorotriesters, phosphoramidates, boranophosphates, methylphosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs), locked-nucleic acids (LNAs), and the like.

[0072] "Percent identity:" To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.,* gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino acid or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = number of identical overlapping positions/total number of positions x 100%). In one embodiment, the two sequences are the same length.

[0073] The determination of percent identity between two sequences can also be accomplished using a mathematical algorithm. One non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:2264-2268, modified as in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci. U.S.A. 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., 1990, J. Mol. Biol. 215:403. BLAST nucleotide searches can be performed with the NBLAST nucleotide program parameters set, *e.g.,* for score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acids described herein. BLAST protein searches can be performed with the XBLAST program parameters set, *e.g.,* to score-50, wordlength = 3 to obtain amino acid sequences homologous to a protein described herein. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., 1997,

Nucleic Acids Res. 25:3389-3402. Alternatively, PSI-BLAST can be used to perform an iterated search which detects distant relationships between molecules (Id.). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.,* of XBLAST and NBLAST) can be used (see, *e.g.,* the NCBI website). Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988, CABIOS 4: 11-17. Such an algorithm is incorporated in the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM 120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

[0074] The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

[0075] As used herein, the terms "prevent," "preventing" and "prevention" in the context of the administration of a therapy(ies) to a subject refer to a prophylactic effect that results from the administration of a therapy or a combination of therapies. In a specific embodiment, the terms "prevent," "preventing" and "prevention" in the context of the administration of a therapy(ies) to a subject to prevent an influenza virus disease refer to one or more of the following effects resulting from the administration of a therapy or a combination of therapies: (i) the inhibition or reduction in the development or onset of an influenza virus disease or a symptom thereof (*e.g.,* fever, myalgia, edema, inflammatory infiltrates); (ii) the inhibition or reduction in the recurrence of an influenza virus disease or a symptom associated therewith; and (iii) the reduction or inhibition in influenza virus infection and/or replication.

[0076] In another specific embodiment, the terms "prevent", "preventing" and "prevention" in the context of the administration of a therapy(ies) to a subject to prevent an influenza virus infection refer to one or more of the following effects resulting from the administration of a therapy or a combination of therapies: (i) the reduction or inhibition of the spread of influenza virus from one cell to another cell; (ii) the reduction or inhibition of the spread of influenza virus from one organ or tissue to another organ or tissue; and/or (iii) the reduction or inhibition of the spread of influenza virus from one region of an organ or tissue to another region of the organ or tissue (*e.g.,* the reduction in the spread of influenza virus from the upper to the lower respiratory tract).

[0077] As used herein, the term "3' proximal" in the context of an open reading frame of an influenza virus gene segment refers to the nucleotides beginning from the start codon of the open reading frame towards the 5' end of the open reading frame. In certain embodiments, the term "3' proximal nucleotides" refers to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleotides within the first 20 to 250 nucleotides of an open reading frame beginning from the start codon towards the 5' end of the open reading frame.

[0078] As used herein, the term "3' proximal coding region" in context of an influenza virus gene segment refers to the first 5 to 450 nucleotides from the 3' end of the coding region of an influenza virus gene segment, or any integer between 5 and 450. In a specific embodiment, the 3' proximal coding region sequence refers to the first 5 to 25 nucleotides from the 3' end of the coding region of an influenza virus gene segment, or any integer between 5 and 25. In another embodiment, the 3' proximal coding region sequence refers to the first 25 to 50 nucleotides from the 3' end of the coding region of an influenza virus gene segment, or any integer between 25 and 50. In another embodiment, the 3' proximal coding region sequence refers to the first 50 to 100 nucleotides from the 3' end of the coding region of an influenza virus gene segment, or any integer between 50 and 100. In another embodiment, the 3' proximal coding region sequence refers to the first 50 to 150 nucleotides from the 3' end of the coding region of an influenza virus gene segment, or any integer between 50 and 150. In another embodiment, the 3' proximal coding region sequence refers to the first 100 to 250 nucleotides from the 3' end of the coding region of an influenza virus gene segment, or any integer between 100 and 250.

[0079] As used herein, the term "3' termini" in the context of an open reading of an influenza virus gene segment refers to the first 20 to 250 nucleotides beginning from the start codon of the open reading frame towards the 5' end of the open reading frame.

[0080] As used herein, the term "5' proximal" in the context of an open reading frame of an influenza virus gene segment refers to the nucleotides beginning from the stop codon of the open reading frame towards the 3' end of the open reading frame. In certain embodiments, the term "5' proximal nucleotides" refers to 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more nucleotides within the first 30 to 250 nucleotides of an open reading frame beginning from the stop codon towards the 3' end of the open reading frame.

[0081] As used herein, the term "5' proximal coding region" in context of an influenza virus gene segment refers to the first 5 to 450 nucleotides from the 5' end of the coding region of an influenza virus gene segment, or any integer between 5 and 450. In a specific embodiment, the 5' proximal coding region sequence refers to the first 5 to 25 nucleotides from the 5' end of the coding region of an influenza virus gene segment, or any integer between 5 and 25. In another embodiment, the 5' proximal coding region sequence refers to the first 25 to 50 nucleotides from the 5' end of the coding region of an influenza virus gene segment, or any integer between 25 and 50. In another embodiment, the 5' proximal coding region sequence refers to the first 50 to 100 nucleotides from the 5' end of the coding region of an influenza virus gene segment, or any integer between 50 and 100. In another embodiment, the 5' proximal coding region sequence refers to the first 50 to 150 nucleotides from the 5' end of the coding region of an influenza virus gene segment, or any

integer between 50 and 150. In another embodiment, the 5' proximal coding region sequence refers to the first 100 to 250 nucleotides from the 5' end of the coding region of an influenza virus gene segment, or any integer between 100 and 250.

[0082] As used herein, the term "5' termini" in the context of an open reading frame of an influenza virus gene segment refers to the first 30 to 250 nucleotides beginning from the stop codon of the open reading frame towards the 3' end of the open reading frame.

[0083] As used herein, the terms "subject" and "patient" are used interchangeably to refer to an animal (*e.g.,* birds, reptiles, and mammals). In a specific embodiment, a subject is a bird. In another embodiment, a subject is a mammal including a non-primate (*e.g.,* a camel, donkey, zebra, cow, pig, horse, goat, sheep, cat, dog, rat, and mouse) and a primate (*e.g.,* a monkey, chimpanzee, and a human). In another embodiment, a subject is a human. In another embodiment, a subject is a human infant. In another embodiment, a subject is a human child. In another embodiment, the subject is a human adult. In another embodiment, a subject is an elderly human. In another embodiment, a subject is a non-human animal (e.g., a non-human mammal or a bird).

[0084] As used herein, the terms "therapies" and "therapy" can refer to any protocol(s), method(s), compound(s), composition(s), formulation(s), and/or agent(s) that can be used in the prevention or treatment of a viral infection or a disease or symptom associated therewith. In certain embodiments, the terms "therapies" and "therapy" refer to biological therapy, supportive therapy, and/or other therapies useful in treatment or prevention of a viral infection or a disease or symptom associated therewith known to one of skill in the art. In some embodiments, the term "therapy" refers to an immunogenic composition (*e.g.,* an influenza virus vaccine).

[0085] As used herein, the terms "treat," "treatment," and "treating" in the context of the administration of a therapy(ies) to a subject refer a beneficial or therapeutic effect resulting from the administration of a therapy or a combination of therapies. In specific embodiments, such terms refer to one, two, three, four, five or more of the following effects resulting from the administration of a therapy or a combination of therapies: (i) reduction or amelioration in the severity of an influenza virus infection, an influenza virus disease or symptom associated therewith; (ii) reduction in the duration of an influenza virus infection, an influenza virus disease or symptom associated therewith; (iii) prevention of the progression of an influenza virus infection, an influenza virus disease or symptom associated therewith; (iv) regression of an influenza virus infection, an influenza virus disease or symptom associated therewith; (v) prevention of the development or onset of an influenza virus infection, an influenza virus disease or symptom associated therewith; (vi) prevention of the recurrence of an influenza virus infection, an influenza virus disease or symptom associated therewith; (vii) reduction or prevention of the spread of an influenza virus from one cell to another cell, one tissue to another tissue, or one organ to another organ; (viii) prevention or reduction of the spread/transmission of an influenza virus from one subject to another subject; (ix) reduction in organ failure associated with an influenza virus infection or influenza virus disease; (x) reduction in the hospitalization of a subject; (xi) reduction in the hospitalization length; (xii) an increase in the survival of a subject with an influenza virus infection or a disease associated therewith; (xiii) elimination of an influenza virus infection or a disease associated therewith; (xiv) inhibition or reduction in influenza virus replication; (xv) inhibition or reduction in the binding or fusion of influenza virus to a host cell(s); (xvi) inhibition or reduction in the entry of an influenza virus into a host cell(s); (xvii) inhibition or reduction of replication of the influenza virus genome; (xviii) inhibition or reduction in the synthesis of influenza virus proteins; (xix) inhibition or reduction in the assembly of influenza virus particles; (xx) inhibition or reduction in the release of influenza virus particles from a host cell(s); (xxi) reduction in influenza virus titer; (xxii) the reduction in the number of symptoms associated with an influenza virus infection or an influenza virus disease; (xxiii) enhancement, improvement, supplementation, complementation, or augmentation of the prophylactic or therapeutic effect(s) of another therapy; (xxiv) prevention of the onset or progression of a secondary infection associated with an influenza virus infection; and/or (xxv) prevention of the onset or diminution of disease severity of bacterial pneumonias occurring secondary to influenza virus infections.

[0086] As used herein, the term "type of influenza virus gene segment(s)" refers to an HA, NA, NS, PB1, PB2, PA, M, or NP gene segment from an influenza virus.

[0087] As used herein, in some embodiments, the term "wild-type" in the context of a virus refers to the types of viruses that are prevalent, circulating and naturally producing typical outbreaks of disease.

4. BRIEF DESCRIPTION OF THE FIGURES

[0088]

Figures. 1A-1B. PB2 Packaging Sequences of PR8. (A) Nucleotide sequence of 3' non-coding region (NCR) (SEQ ID NO:1) and 3' proximal coding region sequence (SEQ ID NO:2) of influenza PR8 virus with an NheI restriction enzyme recognition site (SEQ ID NO:3). The 3' NCR is shaded and the 3' proximal coding region sequence is underlined. (B) Nucleotide sequence of 5' NCR (SEQ ID NO:4) and 5' proximal coding region sequence (SEQ ID NO:5) of influenza PR8 virus with an XhoI restriction enzyme recognition site (SEQ ID NO:6). The 5' NCR is shaded

and the 5' proximal coding region sequence is underlined. Certain capitalized letters represent mutations introduced into the sequence to delete to ATG initiation codon. Additional capitalized letters are found within the XhoI and NheI restriction enzyme recognition sites.

Figures. 2A-2B. PB1 Packaging Sequences of PR8. (A) Nucleotide sequence of 3' NCR (SEQ ID NO:7) and 3' proximal coding region sequence (SEQ ID NO:8) of influenza PR8 virus with an NheI restriction enzyme recognition site (SEQ ID NO:9). The 3' NCR is shaded and the 3' proximal coding region sequence is underlined. (B) Nucleotide sequence of 5' NCR (SEQ ID NO:10) and 5' proximal coding region sequence (SEQ ID NO:11) of influenza PR8 virus with an XhoI restriction enzyme recognition site (SEQ ID NO:12). The 5' NCR is shaded and the 5' proximal coding region sequence is underlined. Certain capitalized letters represent mutations introduced into the sequence to delete to ATG initiation codon. Additional capitalized letters are found within the XhoI and NheI restriction enzyme recognition sites.

Figures. 3A-3B. PA Packaging Sequences of PR8. (A) Nucleotide sequence of 3' NCR (SEQ ID NO:13) and 3' proximal coding region sequence (SEQ ID NO:14) of influenza PR8 virus with an NheI restriction enzyme recognition site (SEQ ID NO: 15). The 3' NCR is shaded and the 3' proximal coding region sequence is underlined. (B) Nucleotide sequence of 5' NCR (SEQ ID NO:16) and 5' proximal coding region sequence (SEQ ID NO:17) of influenza PR8 virus with an XhoI restriction enzyme recognition site (SEQ ID NO:18). The 5' NCR is shaded and the 5' proximal coding region sequence is underlined. Certain capitalized letters represent mutations introduced into the sequence to delete to ATG initiation codon. Additional capitalized letters are found within the XhoI and NheI restriction enzyme recognition sites.

Figures. 4A-4B. HA Packaging Sequences of PR8. (A) Nucleotide sequence of 3' NCR (SEQ ID NO:19) and 3' proximal coding region sequence (SEQ ID NO:20) of influenza PR8 virus with an NheI restriction enzyme recognition site (SEQ ID NO:21). The 3' NCR is shaded and the 3' proximal coding region sequence is underlined. (B) Nucleotide sequence of 5' NCR (SEQ ID NO:22) and 5' proximal coding region sequence (SEQ ID NO:23) of influenza PR8 virus with an XhoI restriction enzyme recognition site(SEQ ID NO:24). The 5' NCR is shaded and the 5' proximal coding region sequence is underlined. Certain capitalized letters represent mutations introduced into the sequence to delete to ATG initiation codon. Additional capitalized letters are found within the XhoI and NheI restriction enzyme recognition sites.

Figures. 5A-5B. NP Packaging Sequences of PR8. (A) Nucleotide sequence of 3' NCR (SEQ ID NO:25) and 3' proximal coding region sequence (SEQ ID NO:26) of influenza PR8 virus with an NheI restriction enzyme recognition site(SEQ ID NO:27). The 3' NCR is shaded and the 3' proximal coding region sequence is underlined. (B) Nucleotide sequence of 5' non-coding region NCR (SEQ ID NO:28) and 5' proximal coding region sequence (SEQ ID NO:29) of influenza PR8 virus with an XhoI restriction enzyme recognition site (SEQ ID NO:30). The 5' NCR is shaded and the 5' proximal coding region sequence is underlined. Certain capitalized letters represent mutations introduced into the sequence to delete to ATG initiation codon. Additional capitalized letters are found within the XhoI and NheI restriction enzyme recognition sites.

Figures. 6A-6B. NA Packaging Sequences of PR8. (A) Nucleotide sequence of 3' NCR (SEQ ID NO:31) and 3' proximal coding region sequence (SEQ ID NO:32)of influenza PR8 virus with an NheI restriction enzyme recognition site (SEQ ID NO:33). The 3' NCR is shaded and the 3' proximal coding region sequence is underlined. (B) Nucleotide sequence of 5' NCR (SEQ ID NO:34) and 5' proximal coding region sequence (SEQ ID NO:35) of influenza PR8 virus with an XhoI restriction enzyme recognition site (SEQ ID NO:36). The 5' NCR is shaded and the 5' proximal coding region sequence is underlined. Certain capitalized letters represent mutations introduced into the sequence to delete to ATG initiation codon. Additional capitalized letters are found within the XhoI and NheI restriction enzyme recognition sites.

Figures. 7A-7B. M Packaging Sequences of PR8. (A) Nucleotide sequence of 3' NCR (SEQ ID NO:37) and 3' proximal coding region sequence (SEQ ID NO:38) of influenza PR8 virus with an NheI restriction enzyme recognition site (SEQ ID NO:39). The 3' NCR is shaded and the 3' proximal coding region sequence is underlined. (B) Nucleotide sequence of 5' NCR (SEQ ID NO:40) and 5' proximal coding region sequence (SEQ ID NO:41) of influenza PR8 virus with an XhoI restriction enzyme recognition site (SEQ ID NO:42). The 5' NCR is shaded and the 5' proximal coding region sequence is underlined. In Fig. 7A, the capitalized letter at position 52 represents the mutation introduced into the sequence in order to eliminate the mRNA 5' splice site. Other capitalized letters represent mutations introduced into the sequence to delete to ATG initiation codon or are found within the XhoI and NheI restriction enzyme recognition sites.

Figures. 8A-8B. NS Packaging Sequences of PR8. (A) Nucleotide sequence of 3' NCR (SEQ ID NO:43) and 3' proximal coding region sequence (SEQ ID NO:44) of influenza PR8 virus with an NheI restriction enzyme recognition site (SEQ ID NO:45). The 3' NCR is shaded and the 3' proximal coding region sequence is underlined. (B) Nucleotide sequence of 5' NCR (SEQ ID NO:46) and 5' proximal coding region sequence (SEQ ID NO:47) of influenza PR8 virus with an XhoI restriction enzyme recognition site (SEQ ID NO:48). The 5' NCR is shaded and the 5' proximal coding region sequence is underlined. In Fig. 8A, the capitalized letter at position 57 represents the mutation intro-

duced into the sequencer in order to eliminate the distal 5' splice site. Other capitalized letters represent mutations introduced into the sequence to delete to ATG initiation codon or are found within the XhoI and NheI restriction enzyme recognition sites.

Figures. 9A-9B. Serial silent mutations introduced into the open reading frame region (ORF) packaging sequences for PB2. (A) Wild-type ORF 3' termini sequence (SEQ ID NO:49). (B) Mutated ORF 3' termini sequence (SEQ ID NO:50). (C) Wild-type ORF 5' termini sequence (SEQ ID NO:51). (D) Mutated ORF 5' termini sequence (SEQ ID NO:52).

Figures. 10A-10B. Serial silent mutations introduced into the open reading frame region (ORF) packaging sequences for PB1. (A) Wild-type ORF 3' termini sequence (SEQ ID NO:53). (B) Mutated ORF 3' termini sequence (SEQ ID NO:54). (C) Wild-type ORF 5' termini sequence (SEQ ID NO:55). (D) Mutated ORF 5' termini sequence (SEQ ID NO:56).

Figures. 11A-11B. Serial silent mutations introduced into the open reading frame region (ORF) packaging sequences for PA. (A) Wild-type ORF 3' termini sequence (SEQ ID NO:57). (B) Mutated ORF 3' termini sequence (SEQ ID NO:58). (C) Wild-type ORF 5' termini sequence (SEQ ID NO:59). (D) Mutated ORF 5' termini sequence (SEQ ID NO:60).

Figures. 12A-12B. Serial silent mutations introduced into the open reading frame region (ORF) packaging sequences for HA. (A) Wild-type ORF 3' termini sequence (SEQ ID NO:61). (B) Mutated ORF 3' termini sequence (SEQ ID NO:62). (C) Wild-type ORF 5' termini sequence (SEQ ID NO:63). (D) Mutated ORF 5' termini sequence (SEQ ID NO:64).

Figures. 13A-13B. Serial silent mutations introduced into the open reading frame region (ORF) packaging sequences for NP. (A) Wild-type ORF 3' termini sequence (SEQ ID NO:65). (B) Mutated ORF 3' termini sequence (SEQ ID NO:66). (C) Wild-type ORF 5' termini sequence (SEQ ID NO:67). (D) Mutated ORF 5' termini sequence (SEQ ID NO:68).

Figures. 14A-14B. Serial silent mutations introduced into the open reading frame region (ORF) packaging sequences for NA. (A) Wild-type ORF 3' termini sequence (SEQ ID NO:69). (B) Mutated ORF 3' termini sequence (SEQ ID NO:70). (C) Wild-type ORF 5' termini sequence (SEQ ID NO:71). (D) Mutated ORF 5' termini sequence (SEQ ID NO:72).

Figures. 15A-15B. Serial silent mutations introduced into the open reading frame region (ORF) packaging sequences for M. (A) Wild-type ORF 3' termini sequence (SEQ ID NO:73). (B) Mutated ORF 3' termini sequence (SEQ ID NO:74). (C) Wild-type ORF 5' termini sequence (SEQ ID NO:75). (D) Mutated ORF 5' termini sequence (SEQ ID NO:76).

Figures. 16A-16B. Serial silent mutations introduced into the open reading frame region (ORF) packaging sequences for NS. (A) Wild-type ORF 3' termini sequence (SEQ ID NO:77). (B) Mutated ORF 3' termini sequence (SEQ ID NO:78). (C) Wild-type ORF 5' termini sequence (SEQ ID NO:79). (D) Mutated ORF 5' termini sequence (SEQ ID NO:80).

Figure. 17. Influenza virus A/WSN/33 HA gene segment (GenBank No. J02176; GI: 324199; SEQ ID NO:84). The nucleotide sequence of the 3' NCR (SEQ ID NO:81) is underlined, the nucleotide sequence of the HA open reading frame (SEQ ID NO:82) is in plain text, and the nucleotide sequence of the 5' NCR (SEQ ID NO:83) is double underlined.

Figure. 18. Influenza virus A/WSN/33 NA gene segment (GenBank No. J02177; GI: 324481; SEQ ID NO:88). The nucleotide sequence of the 3' NCR (SEQ ID NO:85) is underlined, the nucleotide sequence of the NA open reading frame (SEQ ID NO:86) is in plain text, and the nucleotide sequence of the 5' NCR (SEQ ID NO:87) is double underlined.

Figure. 19. Influenza virus A/WSN/33 M gene segment (GenBank No. L25814; GI: 414302; SEQ ID NO:92). The nucleotide sequence of the 3' NCR (SEQ ID NO:89) is underlined, the nucleotide sequence of the M1/M2 open reading frame (SEQ ID NO:90) is in plain text, and the nucleotide sequence of the 5' NCR (SEQ ID NO:91) is double underlined. The open reading frame for M1 is from nucleotides 26 to 784. The open reading frame for M2 is from nucleotides 26 to 51 of exon 1 and nucleotides 740 to 1007 of exon 2.

Figure. 20. Influenza virus A/WSN/33 NS gene segment (GenBank No. Z21498; GI: 296585; SEQ ID NO:96). The nucleotide sequence of the 3' NCR (SEQ ID NO:93) is underlined, the nucleotide sequence of the NS1/NS2 open reading frame (SEQ ID NO:94) is in plain text, and the nucleotide sequence of the 5' NCR (SEQ ID NO:95) is double underlined. The open reading frame for NS1 is from nucleotides 27 to 719. The open reading frame for NS2 is from nucleotides 27 to 56 of exon 1 and nucleotides 529 to 864.

Figure. 21. Influenza virus A/WSN/33 PA gene segment (GenBank No. X17336; GI: 60812; SEQ ID NO:100). The nucleotide sequence of the 3' NCR (SEQ ID NO:97) is underlined, the nucleotide sequence of the PA open reading frame (SEQ ID NO:98) is in plain text, and the nucleotide sequence of the 5' NCR (SEQ ID NO:99) is double underlined.

Figure. 22. Influenza virus A/WSN/33 PB1 gene segment (GenBank No. J02178; GI: 324899; SEQ ID NO:104). The nucleotide sequence of the 3' NCR (SEQ ID NO:101) is underlined, the nucleotide sequence of the PB1 open reading frame (SEQ ID NO:102) is in plain text, and the nucleotide sequence of the 5' NCR (SEQ ID NO:103) is double underlined.

Figure. 23. Influenza virus A/WSN/33 PB2 gene segment (GenBank No. J02179; GI: 324913; SEQ ID NO:108). The nucleotide sequence of the 3' NCR (SEQ ID NO:105) is underlined, the nucleotide sequence of the PB2 open reading frame (SEQ ID NO:106) is in plain text, and the nucleotide sequence of the 5' NCR (SEQ ID NO:107) is double underlined.

Figure. 24. Influenza virus A/WSN/33 NP gene segment (GenBank No. M30746; GI: 324676; SEQ ID NO:112). The nucleotide sequence of the 3' NCR (SEQ ID NO:109) is underlined, the nucleotide sequence of the NP open reading frame (SEQ ID NO:110) is in plain text, and the nucleotide sequence of the 5' NCR (SEQ ID NO:111) is double underlined.

Figures. 25A-25F. Generation of the recombinant Swap(wt) virus carrying HA and NS chimeric segments which can independently reassort. (A) NS-HAwt-NS and HA-NSwt-HA constructs. The A/PR/8/34 HA wild type (HAwt) ORF (hatched) was flanked by the NS 3', 5' NCRs and the 77 nt, 102 nt of NS ORF packaging signals (in red), generating the 1941 nt long NS-HAwt-NS construct; likewise, the NS wild type (NSwt) ORF (straight lines) was flanked by the HA 3', 5' NCRs and the 67 nt, 105 nt of HA ORF packaging signals (hatched), generating the 1099 nt long HA-NSwt-HA construct. The ATGs (in positive sense) upstream of the HA and NS translation start codons were all mutated to TTGs (in positive sense). The 5' splice on the 77 nt part of NS packaging signals in the NS-HAwt-NS construct was also mutated. (B) Genome structure of the Swap(wt) virus. Six A/PR/8/34 ambisense plasmids (Gao Q, Brydon EW, Palese P (2008) A seven-segmented influenza A virus expressing the influenza C virus glycoprotein HEF. J Virol 82:6419-6426, Quinlivan M, et al. (2005) Attenuation of equine influenza viruses through truncations of the NS1 protein. J Virol 79:8431-8439, Kopecky-Bromberg SA, et al. (2009) Alpha-C-galactosylceramide as an adjuvant for a live attenuated influenza virus vaccine. Vaccine 27:3766-3774), and the NS-HAwt-NS and HA-NSwt-HA constructs were used to generate the Swap(wt) virus. (Sequencing of the NS-HAwt-NS RNA segment revealed one G81U mutation in the 3' end. No nucleotide changes were identified for the HA-NSwt-HA RNA segment). (C) Genome structure of the Reassortant(NS) virus which contains seven A/PR/8/34 RNAs and the HA-NSwt-HA RNA. (D) Genome structure of the Reassortant(HA) virus which contains seven A/PR/8/34 RNAs and the NS-HAwt-NS segment. (E) Immunostaining of the plaques formed in MDCK cells by the recombinant viruses. (F) Growth rates of the recombinant viruses in eggs at 37°C.

Figures 26A-26E. Generation of the recombinant Swap(mut) virus carrying HA and NS chimeric segments which can not independently reassort. (A) NS-HAmut-NS and HA-NSmut-HA constructs. The strategy was the same as that described in Figure 17A, except that the ORF region contained serial synonymous mutations: the NS-HAmut-NS construct carried 22 and 45 nt changes at the 3' and 5' ends, respectively; the HA-NSmut-HA construct had 12 and15 nt changes in the NS ORF. (B) Genome structure of the Swap(mut) virus. The genomic composition is similar to that of the Swap(wt) virus (Figure 17B), except that the NS-HAmut-NS and HA-NSmut-HA constructs were substituted for rescue. [Sequencing the NS-HAmut-NS RNA of the Swap(mut) virus revealed eight A to G mutations in the 3' end. The sequence of the 3' end 130 nt of the NS-HAmut-NS RNA is: 3'-ucguuuucgucccacuguuucuguauGaccuagguuugugacacaguucgGGagucgaucuaacgGGagaaaccga acaggcguuugcucaacgucugguucucgGucguacuuucgcuuGGacaaucaa (SEQ ID NO:113; capitalized Gs designate the changes observed in virus RNA). For the HA-NSmut-HA RNA segment, two conversions on the NS 3' ORF region were observed: A122G, which results in a Val to Ala amino acid change; and U318C, which is silent.] (C) Plaque phenotype of the Swap(mut) virus in MDCK cells. (D) Growth rates of the recombinant viruses in 10-day-old embryonated chicken eggs at 37°C. (E) Failure to rescue two hypothetical reassortant viruses. The experiment on the left used seven A/PR/8/34 plasmids (Gao Q, Brydon EW, Palese P (2008) A seven-segmented influenza A virus expressing the influenza C virus glycoprotein HEF. J Virol 82:6419-6426, Quinlivan M, et al. (2005) Attenuation of equine influenza viruses through truncations of the NS1 protein. J Virol 79:8431-8439, Kopecky-Bromberg SA, et al. (2009) Alpha-C-galactosylceramide as an adjuvant for a live attenuated influenza virus vaccine. Vaccine 27:3766-3774) and the HA-NSmut-HA construct, and the one on the right used seven A/PR/8/34 plasmids (Gao Q, Brydon EW, Palese P (2008) A seven-segmented influenza A virus expressing the influenza C virus glycoprotein HEF. J Virol 82:6419-6426, Quinlivan M, et al. (2005) Attenuation of equine influenza viruses through truncations of the NS1 protein. J Virol 79:8431-8439, Kopecky-Bromberg SA, et al. (2009) Alpha-C-galactosylceramide as an adjuvant for a live attenuated influenza virus vaccine. Vaccine 27:3766-3774) and the NS-HAmut-NS.

Figures 27A-27E. Analyzing the vRNA genome packaging efficiency of the recombinant viruses. Five recombinant viruses [rA/PR/8/34 (A), Swap(wt) (B), Reassortant(NS) (C), Reassortant(HA) (D) and Swap(mut) (E)] were grown in eggs at 37°C and purified viral RNA was separated (0.5 μg/lane) on a 2.8% acrylamide gel and visualized by silver staining. The RNA from the rA/PR/8/34 (A) and Swap(mut) (E) viruses was separated on one gel, and the RNA from the other three viruses [Swap(wt) (B), Reassortant(NS) (C) and Reassortant(HA) (D)] was separated on another gel.

Figures 28A-28E. The chimeric NS segment of the Swap(wt), but not of Swap(mut), virus can reassort in infected cells. (A) Diagram of the co-infection experiments. (B) RT-PCR primer design to detect the chimeric and wild type HA segments. The RT-PCR products are 824 bp in length for the NS-HAwt-NS or NS-HAmut-NS segments and

747 bp for the wild type HA. (C) RT-PCR primer design to detect the chimeric and wild type NS segments. The RT-PCR products for the chimeric and wild type NS segments are 405 and 326 bp long, respectively. (D) The Swap(wt) and rA/PR/8/34 viruses co-infection experiment. 24 single plaques were characterized by RT-PCR (10 shown in the gel) using primers indicated in (B) and (C). The rA/PR/8/34 and Swap(wt) viruses were used for RT-PCR control (2nd and 3rd lane). M, marker. (E) The Swap(mut) and rA/PR/8/34 co-infection experiment. 48 single plaques were characterized by RT-PCR (10 shown in the gel). The bands below the wild type or chimeric NS PCR products were artificial by-products of the PCR reaction.

Figures 29A-29H. Generation of influenza viruses with a ninth GFP segment. (A) Generation of NA-PB1mut-NA, NA-PB2mut-NA, NA-PAmut-NA, PB1-GFP-PB1, PB2-GFP-PB2 and PA-GFP-PA constructs. To generate NA-PB1mut-NA, NA-PB2mut-NA, NA-PAmut-NA constructs, the PB1mut, PB2mut or PAmut ORF regions were obtained by PCR and serial silent mutations were introduced into the 3' and 5' proximal regions: 24 and 17 nt for PB1mut; 13 and 36 nt for PB2mut; and 19 and 19 nt for PAmut (see Section 7.1). The PB1mut, PB2mut or PAmut ORFs were then flanked by 179 nt of NA packaging sequences in the 3' end and 215 nt of NA packaging sequences in the 5' end. The ATGs located on the 179 nt of NA packaging sequences were all mutated to TTGs. To generate the PB1-GFP-PB1, PB2-GFP-PB2 and PA-GFP-PA constructs, the GFP ORF region was flanked by the PB1, PB2 and PA packaging sequences, respectively. The PB1 packaging sequences included 153 nt of PB1 3' end and 159 nt of PB1 5' end; The PB2 packaging sequences included 158 nt of PB2 3' end and 169 nt of PB2 5' end; and the PA packaging sequences included 129 nt of PA 3' end and 184 nt of PA 5' end. The ATGs located on the 3' ends of PB1, PB2 and PA packaging sequences were all mutated to TTGs. The translation start and stop codons of each construct are indicated by arrows. (B) Genome structure of -PB1(ps) and -PB1(ps)+GFP viruses. Seven A/PR/8/34 ambisense plasmids (pDZ-PB2, pDZ-PA, pDZ-HA, pDZ-NP, pDZ-NA, pDZ-M, pDZ-NS), and one chimeric construct NA-PB1mut-NA were used to generate the -PB1(ps) virus by using reverse genetics (Fodor et al., 1999, J Virol 73:9679-82; Quinlivan et al., 2005, J Virol 79:8431-9). For the rescue of - PB1(ps)+GFP virus, a ninth PB1-GFP-PB1 construct was included. (C) Genome structure of -PB2(ps)+GFP virus. Similar to the -PB1(ps)+GFP virus in B, the virus contained a chimeric NA-PB2mut-NA segment instead of a wild type PB2, seven A/PR/8/34 segments (PB1, PA, HA, NP, NA, M, NS) and a ninth PB2-GFP-PB2 chimeric segment. The virus lacking a ninth PB2-GFP-PB2 segment was not rescued. (D) Genome structure of -PA(ps) and -PA(ps)+GFP viruses. Similar to -PB1(ps) in B, the -PA(ps) virus contained a chimeric NA-PAmut-NA segment instead of a wild type PA and seven A/PR/8/34 segments (PB2, PB1, HA, NP, NA, M, NS). The -PA(ps)+GFP virus contained a ninth PA-GFP-PA chimeric segment. (E) Growth curves of viruses in 10-day-old embryonated chicken eggs at 37°C. The error bars represent standard deviations. (F) Immunostaining of the plaques formed in MDCK cells by the recombinant viruses two days post infection. (G) GFP expression of recombinant viruses in 293T cells one day post infection (MOI 0.5). The viruses used for infection had been passaged five to ten times in eggs. (H) Hemagglutination assay of viruses grown in 10-day-old embryonated chicken eggs at 37°C.

Figures 30A-30H. Generation of nine-segmented influenza viruses carrying both H1 and H3 subtype HAs. (A) Generation of PB1-HA(HK)-PB1 and PB2-HA(HK)-PB2 constructs. The A/HK/1/68 HA ORF was amplified from a pCAGGS-HK HA plasmid (Wang et al., 2009, PLoS Pathog 6:e1000796) by PCR and used to replace the GFP ORF of PB1-GFP-PB1 and PB2-GFP-PB2 constructs in Fig. 29A, generating the PB1-HA(HK)-PB1 and PB2-HA(HK)-PB2 constructs. (B) Genome structure of -PB1 (ps)+HK HA virus. Similar to -PB1(ps)+GFP virus in Fig. 29B, the virus contained a chimeric NA-PB1mut-NA segment instead of a wild type PB1, seven A/PR/8/34 segments (PB2, PA, HA, NP, NA, M, NS) and a ninth PB1-HA(HK)-PB1 chimeric segment. (C) Genome structure of - PB2(ps)+HK HA virus. The chimeric PB2-HA(HK)-PB2 segment was used to replace the PB2-GFP-PB2 of the -PB2(ps)+GFP virus in Fig. 29C, generating the -PB2(ps)+HK HA virus. (D) Growth curves of viruses in 10-day-old embryonated chicken eggs at 37°C. The error bars represent standard deviations. (E) Western blot to detect the A/PR/8/34 and A/HK/1/68 HAs in purified virions. Viruses [rA/PR/8/34, X31, -PB2(ps)+HK HA and - PB1(ps)+HK HA] were grown in eggs at 37 °C and purified through a 30% sucrose cushion. A Western blot was performed to detect the presence of NP and HA proteins using specific mouse monoclonal antibodies: PY102 for A/PR/8/34 HA0 and HA1 (Reale et al.,1986, J Immunol 137:1352-8), HT103 for A/PR/8/34 NP (O'Neill et al., 1998, Embo J 17:288-96), 66A6 for A/HK/1/68 HA0 and HA1, and 12D1 for A/HK/1/68 HA0 and HA2 (Wang et al., 2009, PLoS Pathog 6:e1000796). (F) Western blot to detect the A/PR/8/34 and A/HK/1/68 HAs in virus infected MDCK cells. MDCK monolayers were infected by viruses [rA/PR/8/34, X31, -PB1(ps)+HK HA and -PB2(ps)+HK HA] at an MOI of 10 to 0.0001. One day post infection, the cells were washed with PBS and harvested using 2 x protein loading buffer [100 mM This-HCl (PH 6.8), 4% sodium dodecyl sulfate, 20% glycerol, 5% β-mercaptoethanol and 0.2% bromophenol blue] and run on a 10% SDS PAGE gel. The A/PR/8/34 HA0, NP, and A/HK/1/68 HA0 were detected by monoclonal antibodies PY102, HT103 and 66A6, respectively (O'Neill et al., 1998, Embo J 17:288-96; Wang et al., 2009, PLoS Pathog 6:e1000796; Wang et al., 2009, PLoS Pathog 6:e1000796). (G) H1/H3 sandwich ELISA to determine whether both H1 and H3 subtype HA proteins were incorporated into the same particles of the -PB1(ps)+HK HA and -PB2(ps)+HK HA viruses (see Section 7.1). The error bars represent standard deviations. (H) Analyzing the vRNA genome packaging efficiency

of the recombinant viruses. Four recombinant viruses [rA/PR/8/34, X31, -PB1(ps)+HK HA and -PB2(ps)+HK HA] were grown in eggs at 37°C and purified viral RNA was separated (0.5 μg/lane) on a 2.8% acrylamide gel and visualized by silver staining. The rRNA band was confirmed based on size and previously reported findings. The identity of an additional band marked with a "?" is unknown.

Figures 31A-31D. Immunization of mice with -PB1(ps)+HK HA virus conferred complete protection from lethal challenges of rA/PR/8/34 and X31 viruses. (A) Growth curves of viruses in 10-day-old embryonated chicken eggs at 37°C. (B) Pathogenicity of -PB1(ps)+HK HA and -PB1(ps)+Luc viruses. Groups of C57BL/6 mice were given PBS, -PB1(ps)+HK HA virus, or the -PB1(ps)+Luc virus, at 10^3 or 10^4 PFU through the intranasal route and observed for two weeks for weight loss and signs of disease. The average body weights of animals in each group are indicated as percentages of the original body weights. (C) rA/PR/8/34 virus challenge experiment. Three weeks after the infection, the groups of mice that received PBS, 10^3 PFU -PB1(ps)+HK HA virus, and 10^3 PFU -PB1(ps)+Luc virus, were challenged intranasally with 100 MLD_{50} of rA/PR/8/34 virus. The mice were then observed daily for two weeks for body weight loss and signs of disease. (D) X31 virus challenge experiment. X31 virus challenge was performed as in (C) except that the groups of mice were challenged by using 33 MLD_{50} of X31 virus instead of rA/PR/8/34 virus. The error bars in A-D represent standard deviations.

Figure 32. Nucleic acid sequences of chimeric gene segments. (A) Nucleic acid sequence of NA-PB1mut-NA (SEQ ID NO:119). (B) Nucleic acid sequence ofNA-PB2mut-NA (SEQ ID NO:120). (C) Nucleic acid sequence of NA-PAmut-NA (SEQ ID NO:121). (D) Nucleic acid sequence of PB1-GFP-PB1 (SEQ ID NO:122). (E) Nucleic acid sequence of PB2-GFP-PB2 (SEQ ID NO:123). (F) Nucleic acid sequence of PA-GFP-PA (SEQ ID NO:124). (G) Nucleic acid sequence of PB1-HA(HK)-PB1 (SEQ ID NO:125). (H) Nucleic acid sequence of PB2-HA(HK)-PB2 (SEQ ID NO:126). (I) Nucleic acid sequence of PB1-Luc-PB1 (SEQ ID NO:127).

Figure 33. The percentage of GFP expressing plaques formed by the - PB2(ps)+GFP and -PB1(ps)+GFP viruses in MDCK cells. Regular plaque assay was performed and immunostaining of the plaques was used to measure the titers of both viruses at passages 1 and 5 in 10-day-old eggs. Mab HT103 (anti-A/PR/8/34 NP) was used in this procedure.

Figure 34. Expression of Renilla luciferase by the -PB1(ps)+Luc virus in MDCK cells. MDCK cells in a 6-well plate were infected by the -PB1(ps)+GFP or - PB 1 (ps)+ Luc virus at an moi of 5. Sixteen hours later, the Renilla luciferase activity was measured using a Rennila luciferase assay system (Promega).

Figure 35. Chimeric gene segments of recombinant influenza virus generated by transfecting 293T cells with chimeric plasmids carrying NA-PB2mut-NA, PB2-PB1mut-PB2, PB1-PAmut-PB1, M-NPmut-M, PA-NAmut-PA, NP-Mmut-NP and 2 plasmids carrying the wild type A/PR/8/34 HA and NS segments.

Figure 36. Chimeric gene segments of recombinant influenza virus generated by transfecting 293T cells with chimeric plasmids carrying NA-PB2mut-NA, PB2-PB1mut-PB2, PB1-PAmut-PB1, NS-HAmut-NS, PA-NAmut-PA, HA-NS-mut-HA and 2 wild type A/PR/8/34 NP and M segments.

Figure 37. Chimeric gene segments of recombinant influenza virus generated by transfecting 293T cells with chimeric plasmids carrying NA-PB2mut-NA, PB2-PB1mut-PB2, PB1-PAmut-PB1, NP-HAmut-NP, NS-NPmut-NS, PA-NAmut-PA, HA-NSmut-HA), and 1 wild type A/PR/8/34 M segments.

Figure 38. Chimeric gene segments of recombinant influenza virus generated by transfecting 293T cells with chimeric plasmids carrying PB2, PB1, PA, HA, NP, PA-NAmut-PA, M, NS segments as well as an NA-GFP ORF-NA or NA-HK HA ORF-NA segment.

5. DETAILED DESCRIPTION

[0089] Described herein are chimeric influenza virus gene segments and nucleic acid sequences encoding such chimeric influenza virus gene segments which are useful in the production of recombinant influenza viruses. In particular, two or more chimeric influenza virus gene segments or complements thereof, or nucleic acid sequences encoding such gene segments or the complements thereof may be used in the production of recombinant influenza viruses. Without being bound by any theory, the two or more chimeric influenza virus gene segments segregate together (*i.e.*, cosegregate) during replication of the recombinant influenza viruses such that the recombinant influenza viruses have a reduced ability to reassort with other influenza viruses (*e.g.*, wild-type influenza viruses) or are unable to reassort with other influenza viruses as determined by techniques known to one skilled in the art. The reduced ability or inability of such recombinant influenza viruses to reassort with other influenza viruses may improve the safety of the recombinant influenza viruses as a live attenuated vaccine. Accordingly, such recombinant influenza viruses may be useful in either the prevention of influenza virus disease, the treatment of influenza virus disease or influenza virus infection, or both.

5.1 NUCLEIC ACIDS

[0090] Provided herein are recombinant influenza viruses comprising at least two nucleic acid sequences that are a

chimera of coding and non-coding regions of two influenza virus gene segments or derivatives thereof, or the complement thereof. Also provided herein are nucleic acid sequences that encode a chimera of coding and non-coding regions of two influenza virus gene segments or derivatives thereof, or the complement thereof. In certain aspects, a nucleic acid sequence provided herein comprises or encodes: (a) packaging signals found in the 3' and the 5' non-coding regions of a first type of influenza virus gene segment or the complements thereof, (b) packaging signals found in the 3' proximal coding region sequence of the first type of influenza virus gene segment or the complement thereof, and the 5' proximal coding region sequence of the first type of influenza virus gene segment or the complement thereof, and (c) an open reading frame or a fragment thereof from a second, different type of influenza virus gene segment, or a complement thereof, wherein the open reading frame contains one, two, three or more silent mutations in the influenza virus packaging signals found in the open reading frame. The first and second types of influenza virus gene segments refer to two different influenza virus gene segments. For example, the first type of influenza virus gene segment may be a hemagglutinin (HA) influenza virus gene segment and the second type of influenza virus gene segment may be an NS influenza virus gene segment. The 3' and the 5' proximal coding regions sequences flank the open reading frame. In certain embodiments, the 3' and the 5' proximal coding region sequences flank the open reading frame and are not translated. The 3' proximal coding region sequence has been mutated so as to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence. In certain embodiments, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site. In some embodiments, the 5' proximal coding region sequence has one or more mutations so as to ensure that the 5' proximal coding region sequence is not translated. The mutations introduced into the open reading frame of the influenza virus gene segment or a fragment thereof are silent mutations.

[0091] Influenza virus gene segment packaging signals are known. In addition, techniques for identifying influenza virus gene segment packaging signals are well known and examples are described in Section 5.8, *infra*. In certain embodiments, a chimeric influenza virus gene segment comprises packaging signals found in the non-coding and coding regions of one type of influenza virus segment that are sufficient to achieve packaging of the chimeric influenza virus gene segment at an efficiency of at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% relative to the packaging of the wild-type influenza virus gene segment that the packaging signals are obtained or derived from. In a specific embodiment, a chimeric influenza virus gene segment comprises packaging signals found in the non-coding and coding regions of one type of influenza virus segment that are sufficient to achieve packaging of the chimeric influenza virus gene segment at an efficiency of at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 98%, or 99% relative to the packaging of the wild-type influenza virus gene segment that the packaging signals are obtained or derived from as determined by acrylamide gel electrophoresis of purified vRNA under the same type of assay conditions. In some embodiments, a chimeric influenza virus gene segment comprises packaging signals found in the non-coding and coding regions of one type of influenza virus segment that are sufficient to achieve packaging of the chimeric influenza virus gene segment at an efficiency of 10% to 50%, 10% to 75%, 10% to 90%, 10% to 95%, 10% to 99.5%, 25% to 50%, 25% to 75%, 25% to 90%, 25% to 99.5%, 50% to 75%, 50% to 90%, or 50% to 99.5% relative to the packaging of the wild-type influenza virus gene segment that the packaging signals are obtained or derived from. In a specific embodiment, a chimeric influenza virus gene segment comprises packaging signals found in the non-coding and coding regions of one type of influenza virus segment that are sufficient to achieve packaging of the chimeric influenza virus gene segment at an efficiency of 10% to 50%, 10% to 75%, 10% to 90%, 10% to 95%, 10% to 99.5%, 25% to 50%, 25% to 75%, 25% to 90%, 25% to 99.5%, 50% to 75%, 50% to 90%, or 50% to 99.5% relative to the packaging of the wild-type influenza virus gene segment that the packaging signals are obtained or derived from as determined by acrylamide gel electrophoresis of purified vRNA under the same type of assay conditions. In other embodiments, a chimeric influenza virus gene segment comprises packaging signals found in the non-coding and coding regions of one type of influenza virus segment that are sufficient to achieve packaging of the chimeric influenza virus gene segment at the same efficiency as the packaging of the wild-type influenza virus gene segment that the packaging signals are obtained or derived from. In a specific embodiment, a chimeric influenza virus gene segment comprises packaging signals found in the non-coding and coding regions of one type of influenza virus segment that are sufficient to achieve packaging of the chimeric influenza virus gene segment at the same efficiency as the packaging of the wild-type influenza virus gene segment that the packaging signals are obtained or derived from as determined by acrylamide gel electrophoresis of purified vRNA under the same type of assay conditions. With respect to the acrylamide gel electrophoreis referenced, virus may be purified and RNA isolated and run on a 2.8% denaturing polyacrylamide gel which may then be stained with a silver staining kit (Invitrogen) (see, *e.g.*, Gao et al., 2008 J. Virol. 82: 6419-6426; Gao et al., 2009 PNAS USA 106(37): 15891-6; and Example 1 herein for a description of such an assay).

[0092] In a specific embodiment, a nucleic acid sequence of the recombinant virus provided herein comprises or

encodes, in the order presented: (a) packaging signals found in the 3' non-coding region of a first type of influenza virus gene segment or a derivative thereof (referred to herein as the "3' NCR1"), or a complement thereof, (b) packaging signals found in the 3' proximal coding region sequence of the first type of influenza virus gene segment or a derivative thereof (referred to herein as the "3' CRS1"), or a complement thereof, (c) an open reading frame or a fragment thereof from a second, different type of influenza virus gene segment or a derivative thereof, wherein the open reading frame contains one, two, three or more silent mutations in the influenza virus packaging signals found in the open reading frame (referred to herein as the "mORF"), or a complement thereof, (d) packaging signals found in the 5' proximal coding region sequence of the first type of influenza virus gene segment or a derivative thereof (referred to herein as the "5' CRS1"), or a complement thereof, and (e) packaging signals found in the 5' non-coding region of the first type of influenza virus gene segment or a derivative thereof (referred to herein as the 5' NCR1"), or a complement thereof. The first and second types of influenza virus gene segments refer to two different influenza virus gene segments. The 3' and the 5' proximal coding region sequences flank the open reading frame. The 3' proximal coding region sequence has been mutated so as to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence. In certain embodiments, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site. In some embodiments, the 5' proximal coding region sequence has one or more mutations so as to ensure that the 5' proximal coding region sequence is not translated. The mutations introduced into the open reading frame of the influenza virus gene segment are silent mutations.

[0093] In one aspect, nucleic acid sequences of the recombinant virus provided herein may comprise or encode a combination of: (i) the following or the complement thereof from one type of influenza virus gene segment: 5' and 3' non-coding regions and a 3' proximal coding region sequence with any start codon eliminated so that it is not translated, and optionally a 5' proximal coding region sequence that is not translated, or both a 3' proximal coding region sequence with any start codon eliminated so that it is not translated and a 5' proximal coding region sequence that is not translated; and (ii) either at least the 3' proximal 20 nucleotides of an open reading frame from a different type of influenza virus gene segment or the complement thereof with one, two three or more mutations, at least the 5' proximal 30 nucleotides of an open reading frame from a different type of influenza virus gene segment or the complement thereof with one, two, three or more mutations, or both the at least 3' proximal 20 nucleotides of an open reading frame and at least the 5' proximal 30 nucleotides of an open reading frame from a different type of influenza virus gene segment or the complement thereof with one, two, three or more mutations. In certain embodiments, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site. In some embodiments, such nucleic acid sequences may be used as a template to engineer in a nucleotide sequence (*e.g.*, a heterologous nucleotide sequence) which is in frame with the 3' proximal 20 nucleotides and/or the 5' proximal 30 nucleotides of the open reading frame from the different type of influenza virus gene segment. In other words, a template chimeric influenza virus gene segment or complement thereof, or a nucleic acid encoding the gene segment or complement thereof may be used as a basis to incorporate a nucleotide sequence (*e.g.*, a heterologous nucleotide sequence) in frame with the 3' and/or 5' proximal nucleotides of the open reading frame of the different type of influenza virus gene segment so that the entire chimeric influenza virus gene segment or complement thereof, or nucleic acid encoding the same does not need to be generated each and every time. The chimeric influenza virus gene segment or complement thereof, or a nucleic acid encoding the gene segment or complement thereof may contain one, two or more restriction enzyme sites that would enable the incorporation of a heterologous nucleotide sequence in frame with the 3' and/or 5' proximal nucleotides of the open reading frame of the different type of influenza virus gene segment. In a specific embodiment, the heterologous nucleotide sequence comprises or encodes coding sequence from a different influenza virus type or strain, or the complement thereof.

[0094] In one embodiment, a nucleic acid sequence of the recombinant virus provided herein is a chimeric influenza virus gene segment that comprises:

(i) a 3' NCR1 which comprises or consists of a 3' non-coding region (NCR) of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of a first of type influenza virus gene segment or a fragment thereof;

(ii) a 3' CRS1 which comprises or consists of a 3' proximal coding region sequence of the first type of influenza virus

gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the first type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;
(iii) a mORF which comprises or consists of (a) at least the 3' proximal 20 nucleotides of an open reading frame of a second type of influenza virus gene segment, or an open reading frame comprising at least the 3' proximal 20 nucleotides of an open reading frame of a second type of influenza virus gene segment and a heterologous nucleotide sequence, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 of the 3' proximal nucleotides of the open reading frame of the second type of influenza virus gene segment have been mutated, and/or (b) at least the 5' proximal 30 nucleotides of an open reading frame of a second type of influenza virus gene segment, or an open reading frame comprising at least the 5' proximal 30 nucleotides of an open reading frame of a second type of influenza virus gene segment and a heterologous nucleotide sequence, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 of the 5' proximal nucleotides of the open reading frame of the second type of influenza virus gene segment have been mutated; and
(iv) a 5' NCR1 which comprises or consists of a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof.

The first and second types of influenza virus gene segments may refer to any two influenza virus gene segments of hemagglutinin (HA), neuraminidase (NA; for influenza A and B viruses), M, NS, PA, PB1, PB2, and NP. For example, the first type of influenza virus gene segment may be an influenza virus HA gene segment and the second type of influenza virus gene segment may be an influenza virus NS gene segment. In a specific embodiment, the mutations introduced into the 3' and/or 5' proximal nucleotides of the open reading frame of the second influenza virus gene segment are silent mutations. In certain embodiments, no additional nucleotides are inserted between (i) to (v). In certain embodiments, the 3'CRS1 is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3'CRS1 is derived from an influenza virus NS gene segment and the 3'CRS1 has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3'CRS1 is derived from an influenza virus M gene segment and the 3'CRS1 has been mutated so as to eliminate the distal 5' splice site.

[0095] In another embodiment, a nucleic acid sequence of the recombinant virus provided herein is a chimeric influenza virus gene segment that comprises:

(i) a 3' NCR1 which comprises or consists of a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof;
(ii) a mORF which comprises or consists of (a) at least the 3' proximal 20 nucleotides of an open reading frame of a second type of influenza virus gene segment, or an open reading frame comprising at least the 3' proximal 20 nucleotides of an open reading frame of a second type of influenza virus gene segment and a heterologous nucleotide sequence, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 of the 3' proximal nucleotides of the open reading frame of the second type of influenza virus gene segment have been mutated, and/or (b) at least the 5' proximal 30 nucleotides of an open reading frame of a second type of influenza virus gene segment, or an open reading frame comprising at least the 5' proximal 30 nucleotides of an open reading frame of a second type of influenza virus gene segment and a heterologous nucleotide sequence, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 of the 5' proximal nucleotides of the open reading frame of the second type of influenza virus gene segment have been mutated;
(iii) a 5' CRS1 which comprises or consists of a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the first type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and
(iv) a 5' NCR1 which comprises or consists of a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of

the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof.

The first and second types of influenza virus gene segments may refer to any two influenza virus gene segments of HA, NA (for influenza A and B viruses), M, NS, PA, PB1, PB2, and NP. For example, the first type of influenza virus gene segment may be an influenza virus HA gene segment and the second type of influenza virus gene segment may be an influenza virus NS gene segment. In a specific embodiment, the mutations introduced into the 3' and 5' proximal nucleotides of the open reading frame of the second influenza virus gene segment are silent mutations. In certain embodiments, no additional nucleotides are inserted between (i) to (v). In certain embodiments, the 3'CRS1 is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3'CRS1 is derived from an influenza virus NS gene segment and the 3'CRS1 has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3'CRS1 is derived from an influenza virus M gene segment and the 3'CRS1 has been mutated so as to eliminate the distal 5' splice site.

[0096] In a specific embodiment, a nucleic acid sequence of the recombinant virus provided herein is a chimeric influenza virus gene segment that comprises:

(i) a 3' NCR1 which comprises or consists of a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof;
(ii) a 3' CRS1 which comprises or consists of a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the first type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;
(iii) a mORF which comprises or consists of (a) at least the 3' proximal 20 nucleotides of an open reading frame of a second type of influenza virus gene segment, or an open reading frame comprising at least the 3' proximal 20 nucleotides of an open reading frame of a second type of influenza virus gene segment and a heterologous nucleotide sequence, wherein the at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 of the 3' proximal nucleotides of the open reading frame of the second type of influenza virus gene segment have been mutated, and/or (b) at least the 5' proximal 30 nucleotides of an open reading frame of a second type of influenza virus gene segment, or an open reading frame comprising at least the 5' proximal 30 nucleotides of an open reading frame of a second type of influenza virus gene segment and a heterologous nucleotide sequence, wherein the at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 of the 5' proximal nucleotides of the open reading frame of the second type of influenza virus gene segment have been mutated;
(iv) a 5' CRS1 which comprises or consists of a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the first type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and
(v) a 5' NCR1 which comprises or consists of a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof.

The first and second types of influenza virus gene segments may refer to any two influenza virus gene segments of HA, NA (for influenza A and B viruses), M, NS, PA, PB1, PB2, and NP. For example, the first type of influenza virus gene segment may be an influenza virus HA gene segment and the second type of influenza virus gene segment may be an influenza virus NS gene segment. In a specific embodiment, the mutations introduced into the 3' and 5' proximal nucleotides of the open reading frame of the second influenza virus gene segment are silent mutations. In certain embodiments, no additional nucleotides are inserted between (i) to (v). In certain embodiments, the 3'CRS1 is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3'CRS1 is derived from an influenza virus NS gene segment and the 3'CRS1 has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3'CRS1 is derived from an influenza virus M gene segment and the 3'CRS1 has been mutated so as to eliminate the

distal 5' splice site.

[0097] In another embodiment, a nucleic acid sequence of the recombinant virus provided herein is a chimeric influenza virus gene segment that comprises:

(i) a 3' NCR1 which comprises or consists of a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof;

(ii) a 3' CRS1 which comprises or consists of a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the first type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;

(iii) a mORF which comprises or consists of an open reading frame of a second type of influenza virus gene segment, or an open reading frame comprising an open reading frame of a second type of influenza virus gene segment and a heterologous nucleotide sequence, wherein 3' and 5' proximal nucleotides of the open reading frame of the second type of influenza virus gene segment have been mutated; and

(iv) a 5' NCR1 which comprises or consists of a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof.

The first and second types of influenza virus gene segments may refer to any two influenza virus gene segments of HA, NA (for influenza A and B viruses), M, NS, PA, PB1, PB2, and NP. For example, the first type of influenza virus gene segment may be an influenza virus HA gene segment and the second type of influenza virus gene segment may be an influenza virus NS gene segment. In a specific embodiment, the mutations introduced into the 3' and 5' proximal nucleotides of the open reading frame of the second influenza virus gene segment are silent mutations. In certain embodiments, no additional nucleotides are inserted between (i) to (v). In certain embodiments, the 3'CRS1 is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3'CRS1 is derived from an influenza virus NS gene segment and the 3'CRS1 has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3'CRS1 is derived from an influenza virus M gene segment and the 3'CRS1 has been mutated so as to eliminate the distal 5' splice site.

[0098] In another embodiment, a nucleic acid sequence of the recombinant virus provided herein is a chimeric influenza virus gene segment that comprises:

(i) a 3' NCR1 which comprises or consists of a 3' non-coding region (NCR) of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof;

(ii) a mORF which comprises or consists of an open reading frame of a second type of influenza virus gene segment, or an open reading frame comprising an open reading frame of a second type of influenza virus gene segment and a heterologous nucleotide sequence, wherein 3' and 5' proximal nucleotides of the open reading frame of the second type of influenza virus gene segment have been mutated;

(iii) a 5' CRS1 which comprises or consists of a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the first type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and

(iv) a 5' NCR1 which comprises or consists of a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under

stringent conditions to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof.

The first and second types of influenza virus gene segments may refer to any two influenza virus gene segments of HA, NA (for influenza A and B viruses), M, NS, PA, PB1, PB2, and NP. For example, the first type of influenza virus gene segment may be an influenza virus HA gene segment and the second type of influenza virus gene segment may be an influenza virus NS gene segment. In a specific embodiment, the mutations introduced into the 3' and 5' proximal nucleotides of the open reading frame of the second influenza virus gene segment are silent mutations. In certain embodiments, no additional nucleotides are inserted between (i) to (v). In certain embodiments, the 3'CRS1 is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3'CRS1 is derived from an influenza virus NS gene segment and the 3'CRS1 has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3'CRS1 is derived from an influenza virus M gene segment and the 3'CRS1 has been mutated so as to eliminate the distal 5' splice site.

[0099] In a specific embodiment, a nucleic acid sequence of the recombinant virus herein is a chimeric influenza virus gene segment that comprises:

(i) a 3' NCR1 which comprises or consists of a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof;
(ii) a 3' CRS1 which comprises or consists of a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the first type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;
(iii) a mORF which comprises or consists of an open reading frame of a second type of influenza virus gene segment, or an open reading frame comprising an open reading frame of a second type of influenza virus gene segment and a heterologous nucleotide sequence, wherein 3' and 5' proximal nucleotides of the open reading frame of the second type of influenza virus gene segment have been mutated;
(iv) a 5' CRS1 which comprises or consists of a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the first type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and
(v) a 5' NCR1 which comprises or consists of a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof.

The first and second types of influenza virus gene segments may refer to any two influenza virus gene segments of HA, NA (for influenza A and B viruses), M, NS, PA, PB1, PB2, and NP. For example, the first type of influenza virus gene segment may be an influenza virus HA gene segment and the second type of influenza virus gene segment may be an influenza virus NS gene segment. In a specific embodiment, the mutations introduced into the 3' and 5' proximal nucleotides of the open reading frame of the second influenza virus gene segment are silent mutations. In certain embodiments, no additional nucleotides are inserted between (i) to (v). In certain embodiments, the 3'CRS1 is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3'CRS1 is derived from an influenza virus NS gene segment and the 3 'CRS 1 has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3'CRS1 is derived from an influenza virus M gene segment and the 3'CRS1 has been mutated so as to eliminate the distal 5' splice site.

[0100] The chimeric influenza virus gene segments described herein may be a chimeric of coding and non-coding regions of any two influenza virus gene segments of HA, NA (for influenza A and B viruses), M, NS, PA, PB1, PB2, and NP or derivatives thereof. The coding and non-coding regions that make up a chimeric influenza virus gene segment may be obtained or derived from the same type of influenza virus or the same strain of influenza virus. The coding and non-coding regions that make up a chimeric influenza virus gene segment may also be obtained or derived from different types of influenza viruses, different subtypes of influenza viruses or different strains of influenza virus. The coding and

non-coding regions that make up a chimeric influenza virus gene segment may be obtained or derived from a seasonal or pandemic strain of influenza virus.

[0101] In one embodiment, the coding and non-coding regions that make up a chimeric influenza virus gene segment are obtained or derived from an influenza A virus (see Section 5.2, *infra,* for examples of influenza A viruses). In another embodiment, the coding and non-regions that make up a chimeric influenza virus gene segment are obtained or derived from the same strain of an influenza A virus. In another embodiment, the coding and non-coding regions that make up a chimeric influenza virus gene segment are obtained or derived from the same HA and/or NA subtype. For example, the coding and non-coding regions may be from an influenza A virus of the H1N1 subtype.

[0102] In a specific embodiment, the 3' and/or 5' NCR from an influenza A virus, influenza B virus, or influenza C virus is of the same strain or subtype; and/or the 3' and/or 5' proximal coding region sequence from an influenza A virus, influenza B virus, or influenza C virus is of the same strain or subtype.

[0103] In another embodiment, the coding and non-coding regions that make up a chimeric influenza virus gene segment are obtained or derived from an influenza B virus (see Section 5.2, *infra,* for examples of influenza B viruses). In another embodiment, the coding and non-regions that make up a chimeric influenza virus gene segment are obtained or derived from the same strain of an influenza B virus. In another embodiment, the coding and non-coding regions that make up a chimeric influenza virus gene segment are obtained or derived from an influenza C virus (see Section 5.2, *infra,* for examples of influenza C viruses). In another embodiment, the coding and non-regions that make up a chimeric influenza virus gene segment are obtained derived from the same strain of an influenza C virus. of the recombinant virus

[0104] The nucleic acid sequence of the recombinant virus provided herein may be in the form of a genomic *(i.e.,* negative sense RNA) or antigenomic *(i.e.,* positive sense RNA) segment. The nucleic acid sequences may also encode a chimeric influenza virus gene segment or the complement thereof. In one embodiment, a nucleic acid sequence is a chimeric influenza virus gene segment. In another embodiment, a nucleic acid sequence comprises the complement of a chimeric influenza virus gene segment described herein. In another embodiment, a nucleic acid sequence encodes a chimeric influenza virus gene segment described herein or the complement thereof.

[0105] In certain embodiments, a nucleic acid sequence that encodes a chimeric influenza virus gene segment described herein or the complement thereof is bicistronic and permits the expression of two sequences. In other words, the nucleic acid sequence encodes for an mORF and another open reading frame (e.g., an open reading frame encoding a heterologous protein). In one embodiment, such a nucleic acid sequence comprises an internal ribosomal entry site (IRES) after the mORF and before the other open reading frame.

[0106] In certain embodiments, a nucleic acid sequence that encodes a chimeric influenza virus gene segmenf of the recombinant virus provided herein or the complement thereof comprises a promoter. Specific examples of promoters include an RNA polymerase I promoter, an RNA polymerase II promoter, an RNA polymerase III promoter, a T7 promoter and a T3 promoter. In a specific embodiment, a nucleic acid sequence that encodes a chimeric influenza virus gene segment or the complement thereof comprises a human RNA polymerase I promoter. In certain embodiments, a nucleic acid sequence that encodes a chimeric influenza virus gene segment or the complement thereof comprises a transcription termination sequence. Specific examples of transcription termination sequences include an RNA polymerase I terminator sequence, an RNA polymerase II terminator sequence, or an RNA polymerase III terminator sequence. In some embodiments, a nucleic acid sequence that encodes a chimeric influenza virus gene segment or the complement thereof comprises a ribozyme recognition sequence. In a specific embodiment, a nucleic acid sequence that encodes a chimeric influenza virus gene segment or the complement thereof comprises an RNA polymerase I promoter sequence and an RNA polymerase I terminator sequence. In certain embodiments, a nucleic acid sequence that encodes a chimeric influenza virus gene segment or the complement thereof comprises an RNA polymerase I promoter, an RNA polymerase I termination sequence, an RNA polymerase II promoter, and a polyadenylation signal.

[0107] In certain cases, a nucleic acid sequence described herein is part of or incorporated into a vector. In a specific case, a nucleic acid sequence described herein is part of or incorporated into a vector that facilitates the production of a chimeric influenza virus gene segment or the complement thereof. In one case, a nucleic acid sequence described herein is part of or incorporated into the pDZ vector (see, e.g., Quinlivan et al., 2005, J. of Virology 79: 8431-8439 for information relating to the pDZ vector). In another case, a nucleic acid sequence described herein is part of or incorporated into the pHW2000 vector (see, e.g., Hoffmann et al., 2000, Proc Natl Acad Sci USA. 97(11):6108-13 for information relating to the pHW2000 vector). In another case, a nucleic acid sequence described herein is part of or incorporated into the pAD3000 vector (see, e.g., Hoffmann et al., 2000, Proc Natl Acad Sci USA. 97(11):6108-13 for information relating to the pAD3000 vector). In another case, a nucleic acid sequence described herein is part of or incorporated into the pAD4000 vector (see, e.g., Wang et al., 2007, J. of Virology 4: 102 for information relating to the pAD4000 vector).

[0108] In some embodiments, a nucleic acid sequence used to produce the recombinant virus provided herein is introduced (e.g., transfected) into a substrate, such as a host cell or an embryonated egg. Thus, in some embodiments, provided herein is a substrate (e.g., host cells or eggs) comprising the recombinant virus comprising a nucleic acid sequence described herein. In other embodiments, a nucleic acid sequence described herein that is part of or incorporated into a vector is introduced (e.g., transfected) into a substrate, such as a host cell or an embryonated egg. Thus, in some

embodiments, provided herein is a substrate (e.g., host cells or eggs) comprising a the recombinant virus comprising nucleic acid sequence described herein that is part of or incorporated into a vector. Host cells and embryonated eggs are known in the art and examples are provided herein, e.g., in Section 5.4, *infra.*

[0109] In certain embodiments, a nucleic acid of the recombinant virus provided herein is propagated in an influenza virus. In certain embodiments, a group of cosegregating chimeric influenza virus gene segments (see Section 5.2, entitled Influenza Virus Comprising Chimeric Influenza Virus Gene Segment) is propagated in an influenza virus.

[0110] In specific aspects, multiple chimeric influenza virus gene segments may be produced to construct the recombinant virus of the invention. Influenza A virus has a total of eight (8) gene segments and a chimeric of two, three, four, five, six, seven or all eight gene segments may be produced. Influenza B virus has a total of eight (8) gene segments and a chimeric of two, three, four, five, six, seven or all eight gene segments may be produced. Influenza C virus has a total of seven (7) gene segments and a chimeric of two, three, four, five, six or all seven gene segments may be produced. In a specific embodiment, two or more chimeric influenza virus gene segments are produced. By way of example and not limitation, two chimeric influenza virus gene segments may be produced, wherein

(a) the first chimeric influenza virus gene segment comprises:

(i) a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof;
(ii) a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the first type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;
(iii) an open reading frame of a second influenza virus gene segment, or an open reading frame comprising an open reading frame of a second type of influenza virus gene segment and a heterologous nucleotide sequence, wherein 3' and 5' proximal nucleotides of the open reading frame of the second type of influenza virus gene segment have been mutated;
(iv) a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the first type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and
(v) a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof; and wherein

(b) the second chimeric influenza virus gene segment comprises:

(i) a 3' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of the second type of influenza virus gene segment or a fragment thereof;
(ii) a 3' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the second type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;
(iii) an open reading frame of the first type of influenza virus gene segment, or an open reading frame comprising an open reading frame of the first type of influenza virus gene segment and a heterologous nucleotide sequence,

wherein 3' and 5' proximal nucleotides of the open reading frame of the first type of influenza virus gene segment have been mutated;

(iv) a 5' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the second type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and

(v) a 5' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the second type of influenza virus gene segment or a fragment thereof.

In certain embodiments, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site.

[0111] In another specific embodiment, three or more chimeric influenza virus gene segments are produced. By way of example and not limitation, three chimeric influenza virus gene segments may be produced, wherein

(a) the first chimeric influenza virus gene segment comprises:

(i) a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof;

(ii) a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the first type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;

(iii) an open reading frame of a third type of influenza virus gene segment, or an open reading frame comprising an open reading frame of a third type of influenza virus gene segment and a heterologous nucleotide sequence, wherein 3' and 5' proximal nucleotides of the open reading frame of the third type of influenza virus gene segment have been mutated;

(iv) a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the first type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and

(v) a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof; and wherein

(b) the second chimeric influenza virus gene segment comprises:

(i) a 3' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of the second type of influenza virus gene segment or a fragment thereof;

(ii) a 3' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the second type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;

(iii) an open reading frame of the first type of influenza virus gene segment, or an open reading frame comprising an open reading frame of the first type of influenza virus gene segment and a heterologous nucleotide sequence, wherein 3' and 5' proximal nucleotides of the open reading frame of the first type of influenza virus gene segment have been mutated;

(iv) a 5' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the second type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and

(v) a 5' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the second type of influenza virus gene segment or a fragment thereof; wherein

(c) the third chimeric influenza virus gene segment comprises:

(i) a 3' NCR of the third type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of the third type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of the third type of influenza virus gene segment or a fragment thereof;

(ii) a 3' proximal coding region sequence of the third type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the third type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the third type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;

(iii) an open reading frame of the second type of influenza virus gene segment, or an open reading frame comprising an open reading frame of the second type of influenza virus gene segment and a heterologous nucleotide sequence, wherein 3' and 5' proximal nucleotides of the open reading frame of the second type of influenza virus gene segment have been mutated;

(iv) a 5' proximal coding region sequence of the third type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the third type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the third type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and

(v) a 5' NCR of the third type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the third type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the third type of influenza virus gene segment or a fragment thereof.

In certain embodiments, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site.

[0112] Techniques for the production or use of the nucleic acids will employ, unless otherwise indicated, routine conventional techniques of molecular biology and recombinant DNA manipulation and production. Any cloning technique

known to the skilled artisan can be used to assemble the nucleic acids described herein and to mutate nucleotides where necessary. Such techniques are well-known and are available to the skilled artisan in laboratory manuals such as Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001). In particular, polymerase chain reaction, restriction enzymes, ligase enzyme, mutagenic primers, and amplification of nucleic acid fragments in vectors can be used to generate the individual elements of the nucleic acids described herein and then to assemble them.

5.1.1. INFLUENZA VIRUS NONCODING REGIONS

[0113] The chimeric influenza virus gene segmentsof the recombinant virus provided herein comprise a 3' NCR1 and a 5' NCR1. A 3' NCR1 comprises or consists of packaging signals found in the 3' non-coding region an influenza virus gene segment or a derivative thereof. In a specific embodiment, a 3' NCR1 comprises or consists of a 3' NCR of an influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of an influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of an influenza virus gene segment or a fragment thereof. A 5' NCR1 comprises or consists of packaging signals found in the 5' non-coding region an influenza virus gene segment or a derivative thereof. In a specific embodiment, a 5' NCR1 comprises or consists of a 5' NCR of an influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of an influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of an influenza virus gene segment or a fragment thereof. In a specific embodiment, the 3' NCR1 and the 5' NCR1 are from the same type of influenza virus gene segment. In other words, the 3' NCR1 and the 5' NCR1 are both from an HA, NA (for influenza A and B viruses), M, NS, PA, PB1, PB2, or NP influenza virus gene segment. The 3' NCR1 and the 5' NCR1 may be from the same type of influenza virus gene segment (HA, NA (for influenza A and B viruses), M, NS, PA, PB1, PB2, or NP) from the same influenza virus strain. For example, the 3' NCR1 and 5' NCR1 may both be from an HA influenza virus gene segment of the same influenza virus strain. Alternatively, the 3' NCR1 and the 5' NCR1 may be from the same type of influenza virus gene segment from two different strains of influenza virus. For example, the 3' NCR1 may be from an HA gene segment of one influenza virus strain and the 5' NCR1 may be from an HA gene segment of a different influenza virus strain.

[0114] In a specific embodiment, a 3' NCR1 and a 5' NCR1 are from the same type of influenza virus gene segment from an influenza A virus (see Section 5.2, *infra,* for examples of influenza A viruses). In other embodiments, a 3' NCR1 and a 5' NCR1 are from the same type of influenza virus gene segment from an influenza B virus (see Section 5.2, *infra,* for examples of influenza B viruses). In other embodiments, a 3' NCR1 and a 5' NCR1 are from the same type of influenza virus gene segment from an influenza C virus (see Section 5.2, *infra,* for examples of influenza C viruses). In some embodiments, a 3' NCR1 and a 5' NCR1 are from an influenza virus gene segment from a pandemic influenza virus. In other embodiments, a 3' NCR1 and a 5' NCR1 are from an influenza virus gene segment from a seasonal influenza virus.

[0115] In certain embodiments, a 3' NCR1 comprises or consists of the entire 3' NCR of an influenza virus gene segment. The 3' NCRs for influenza viruses are known in the art or can readily be determined using standard molecular biology and virology techniques. For example, the 3' NCR for each segment of the influenza A/WSN/33 (WSN) virus is provided in Table 1, *infra.*

Table 1

WSN Gene Segment	Length of 3' NCR	FIG./SEQ ID NO:
HA	32	FIG. 17/ SEQ ID NO: 81
NA	19	FIG. 18/ SEQ ID NO: 85
M	25	FIG. 19/ SEQ ID NO: 89
NS	26	FIG. 20/ SEQ ID NO: 93
PA	24	FIG. 21/ SEQ ID NO: 97
PB1	24	FIG. 22/ SEQ ID NO: 101
PB2	27	FIG. 23/ SEQ ID NO: 105
NP	45	FIG. 24/ SEQ ID NO: 109

[0116] By way of example and not by limitation, provided in Table 2, *infra,* are nucleotide sequences of the 3' NCR for each segment of the influenza A/PR/8/34 (PR8) virus.

Table 2

PR8 Gene Segment	Length of Sequence	FIG./SEQ ID NO:
HA	32	FIG.4/ SEQ ID NO: 19
NA	20	FIG.6/ SEQ ID NO: 31
M	25	FIG.7/ SEQ ID NO: 37
NS	26	FIG.8/ SEQ ID NO: 43
PA	24	FIG.3/ SEQ ID NO: 13
PB1	24	FIG.2/ SEQ ID NO: 7
PB2	27	FIG.1/ SEQ ID NO: 1
NP	45	FIG.5/ SEQ ID NO: 25

[0117] In some embodiments, a 3' NCR1 comprises or consists of a fragment of the 3' NCR of an influenza virus gene segment. In certain embodiments, a 3' NCR1 comprises or consists of 35, 30, 25, 20, 15, 10 or 5 nucleotides or 5 to 10, 5 to 15, 5 to 20, 5 to 25, 5 to 30, 5 to 35, 10 to 15, 10 to 20, 10 to 25, 10 to 30, 10 to 35, 15 to 20, 15 to 25, 15 to 30, 15 to 35, 20 to 25, 20 to 30, 20 to 35, 25 to 30, or 25 to 35 nucleotides of the 3' NCR of an influenza virus gene segment. In some embodiments, a 3' NCR1 comprises or consists of a nucleotide sequence that is at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to the 3' NCR of an influenza virus gene segment. In certain embodiments, a 3' NCR1 comprises or consists of a nucleotide sequence that is 50% to 65%, 60% to 80%, 65% to 90%, 70% to 95%, 80% to 95%, 90% to 99%, 95% to 99% identical to the 3' NCR of an influenza virus gene segment.

[0118] In some embodiments, a 3' NCR1 comprises or consists of a nucleotide sequence that is at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to 35, 30, 25, 20, 15, 10 or 5 contiguous nucleotides or 5 to 10, 5 to 15, 5 to 20, 5 to 25, 5 to 30, 5 to 35, 10 to 15, 10 to 20, 10 to 25, 10 to 30, 10 to 35, 15 to 20, 15 to 25, 15 to 30, 15 to 35, 20 to 25, 20 to 30, 20 to 35, 25 to 30, or 25 to 35 contiguous nucleotides of the 3' NCR of an influenza virus gene segment. In certain embodiments, a 3' NCR1 comprises or consists of a nucleotide sequence that is 50% to 65%, 60% to 80%, 65% to 90%, 70% to 95%, 80% to 95%, 90% to 99%, 95% to 99% identical to 35, 30, 25, 20, 15, 10 or 5 contiguous nucleotides or 5 to 10, 5 to 15, 5 to 20, 5 to 25, 5 to 30, 5 to 35, 10 to 15, 10 to 20, 10 to 25, 10 to 30, 10 to 35, 15 to 20, 15 to 25, 15 to 30, 15 to 35, 20 to 25, 20 to 30, 20 to 35, 25 to 30, or 25 to 35 contiguous nucleotides of the 3' NCR of an influenza virus gene segment

[0119] In some embodiments, a 3' NCR1 comprises or consists of a nucleotide sequence that hybridizes under stringent conditions to the 3' NCR of an influenza virus gene segment. In certain embodiments, a 3' NCR1 comprises or consists of a nucleotide sequence that hybridizes under stringent conditions to a fragment of the 3' NCR of an influenza virus gene segment. In some embodiments, a 3' NCR1 comprises of consists of a nucleotide sequence that hybridizes under stringent conditions to a sequence consisting of 35, 30, 25, 20, 15, 10 or 5 contiguous nucleotides or 5 to 10, 5 to 15, 5 to 20, 5 to 25, 5 to 30, 5 to 35, 10 to 15, 10 to 20, 10 to 25, 10 to 30, 10 to 35, 15 to 20, 15 to 25, 15 to 30, 15 to 35, 20 to 25, 20 to 30, 20 to 35, 25 to 30, or 25 to 35 contiguous nucleotides of the 3' NCR of an influenza virus gene segment.

[0120] In certain embodiments, a 5' NCR1 comprises or consists of the entire 5' NCR of an influenza virus gene segment. The 5' NCRs for influenza viruses are known in the art or can readily be determined using standard molecular biology and virology techniques. For example, the 5' NCR for each segment of the influenza A/WSN/33 (WSN) virus is provided in Table 3, *infra.*

Table 3

WSN Gene Segment	Length of 5' NCR	SEQ ID NO:
HA	45	FIG. 17/ SEQ ID NO: 83
NA	28	FIG. 18/ SEQ ID NO: 87
M	20	FIG. 19/ SEQ ID NO: 91
NS	26	FIG. 20/ SEQ ID NO: 95

(continued)

WSN Gene Segment	Length of 5' NCR	SEQ ID NO:
PA	58	FIG. 21/ SEQ ID NO: 99
PB1	43	FIG. 22/ SEQ ID NO: 103
PB2	34	FIG. 23/ SEQ ID NO: 107
NP	23	FIG. 24/ SEQ ID NO: 111

[0121] By way of example and not by limitation, provided in Table 4, *infra,* are nucleotide sequences of the 5' NCR for each segment of the influenza A/PR/8/34 (PR8) virus.

Table 4

PR8 Gene Segment	Length of Sequence	FIG./SEQ ID NO:
HA	45	FIG.4/ SEQ ID NO: 22
NA	28	FIG.6/ SEQ ID NO: 34
M	20	FIG.7/ SEQ ID NO: 40
NS	26	FIG.8/ SEQ ID NO: 46
PA	58	FIG.3/ SEQ ID NO: 16
PB1	43	FIG.2/ SEQ ID NO: 10
PB2	34	FIG.1/ SEQ ID NO: 4
NP	23	FIG.5/ SEQ ID NO: 28

[0122] In some embodiments, a 5' NCR1 comprises or consists of a fragment of the 5' NCR of an influenza virus gene segment. In certain embodiments, a 5' NCR1 comprises or consists of 35, 30, 25, 20, 15, 10 or 5 nucleotides or 5 to 10, 5 to 15, 5 to 20, 5 to 25, 5 to 30, 5 to 35, 10 to 15, 10 to 20, 10 to 25, 10 to 30, 10 to 35, 15 to 20, 15 to 25, 15 to 30, 15 to 35, 20 to 25, 20 to 30, 20 to 35, 25 to 30, or 25 to 35 nucleotides of the 5' NCR of an influenza virus gene segment. In some embodiments, a 5' NCR1 comprises or consists of a nucleotide sequence that is at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to the 5' NCR of an influenza virus gene segment. In certain embodiments, a 5' NCR1 comprises or consists of a nucleotide sequence that is 50% to 65%, 60% to 80%, 65% to 90%, 70% to 95%, 80% to 95%, 90% to 99%, 95% to 99% identical to the 5' NCR of an influenza virus gene segment.

[0123] In some embodiments, a 5' NCR1 comprises or consists of a nucleotide sequence that is at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to 35, 30, 25, 20, 15, 10 or 5 contiguous nucleotides or 5 to 10, 5 to 15, 5 to 20, 5 to 25, 5 to 30, 5 to 35, 10 to 15, 10 to 20, 10 to 25, 10 to 30, 10 to 35, 15 to 20, 15 to 25, 15 to 30, 15 to 35, 20 to 25, 20 to 30, 20 to 35, 25 to 30, or 25 to 35 contiguous nucleotides of the 5' NCR of an influenza virus gene segment. In certain embodiments, a 5' NCR1 comprises or consists of a nucleotide sequence that is 50% to 65%, 60% to 80%, 65% to 90%, 70% to 95%, 80% to 95%, 90% to 99%, 95% to 99% identical to 35, 30, 25, 20, 15, 10 or 5 contiguous nucleotides or 5 to 10, 5 to 15, 5 to 20, 5 to 25, 5 to 30, 5 to 35, 10 to 15, 10 to 20, 10 to 25, 10 to 30, 10 to 35, 15 to 20, 15 to 25, 15 to 30, 15 to 35, 20 to 25, 20 to 30, 20 to 35, 25 to 30, or 25 to 35 contiguous nucleotides of the 5' NCR of an influenza virus gene segment.

[0124] In some embodiments, a 5' NCR1 comprises or consists of a nucleotide sequence that hybridizes under stringent conditions to the 5' NCR of an influenza virus gene segment. In certain embodiments, a 5' NCR1 comprises or consists of a nucleotide sequence that hybridizes under stringent conditions to a fragment of the 5' NCR of an influenza virus gene segment. In some embodiments, a 5' NCR1 comprises of consists of a nucleotide sequence that hybridizes under stringent conditions to a sequence consisting of 35, 30, 25, 20, 15, 10 or 5 contiguous nucleotides or 5 to 10, 5 to 15, 5 to 20, 5 to 25, 5 to 30, 5 to 35, 10 to 15, 10 to 20, 10 to 25, 10 to 30, 10 to 35, 15 to 20, 15 to 25, 15 to 30, 15 to 35, 20 to 25, 20 to 30, 20 to 35, 25 to 30, or 25 to 35 contiguous nucleotides of the 5' NCR of an influenza virus gene segment.

5.1.2. INFLUENZA VIRUS TERMINAL CODING REGION THAT IS NOT TRANSLATED

[0125] The chimeric influenza virus gene segments of the recombinant virus provided herein comprise both a 3' CRS1 and a 5' CRS1. A 3' CRS1 comprises or consists of packaging signals found in the 3' proximal coding region sequence of an influenza virus gene segment or a derivative thereof. In a specific embodiment, a 3' CRS1 comprises or consists of a 3' proximal coding region sequence of an influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of an influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of an influenza virus gene segment. The 3' proximal coding region sequence is not translated. The 3' proximal coding region sequence has been mutated so as to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence. In certain embodiments, the 3' proximal coding region sequence of an influenza virus gene segment is from an influenza virus NS or M gene segment. In a specific embodiment, the 3' proximal coding region sequence of an influenza virus gene segment is from an influenza virus NS gene segment and the mRNA 5' splice site has been mutated to prevent splicing from occurring. In another specific embodiment, the 3' proximal coding region sequence of an influenza virus gene segment is from an influenza virus M gene segment and the mRNA distal 5' splice site has been mutated to prevent splicing from occurring.

[0126] A 5' CRS1 comprises or consists of packaging signals found in the 5' proximal coding region sequence of an influenza virus gene segment or a derivative thereof. In a specific embodiment, 5' CRS1 comprises or consists of a 5' proximal coding region sequence of an influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of an influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of an influenza virus gene segment. In certain embodiments, the 5' proximal coding region sequence is translated. In other embodiments, the 5' proximal coding region sequence is not translated. In some embodiments, the 5' proximal coding region sequence has one or more mutations so as to ensure that the 5' proximal coding region sequence is not translated.

[0127] In a specific embodiment, the 3' CRS1 and the 5' CRS1 are from the same type of influenza virus gene segment. In other words, the 3' CRS1 and the 5' CRS1 are both from an HA, NA (for influenza A and B viruses), M, NS, PA, PB1, PB2, or NP influenza virus gene segment. The 5' CRS1 and the 5' CRS1 may be from the same type of influenza virus gene segment (HA, NA (for influenza A and B viruses), M, NS, PA, PB1, PB2, or NP) from the same influenza virus strain. For example, the 3' CRS1 and 5' CRS1 may both be from an HA influenza virus gene segment of the same influenza virus strain. Alternatively, the 3' CRS1 and the 5' CRS1 may be from the same type of influenza virus gene segment from two different strains of influenza virus. For example, the 3' CRS1 may be from an HA gene segment of one influenza virus strain and the 5' CRS1 may be from an HA gene segment of a different influenza virus strain.

[0128] In a specific embodiment, a 3' CRS1 and a 5' CRS1 are from the same type of influenza virus gene segment from an influenza A virus (see Section 5.2, *infra,* for examples of influenza A viruses). In other embodiments, a 3' CRS1 and a 5' CRS1 are from the same type of influenza virus gene segment from an influenza B virus (see Section 5.2, *infra,* for examples of influenza B viruses). In other embodiments, a 3' CRS1 and a 5' CRS1 are from the same type of influenza virus gene segment from an influenza C virus (see Section 5.2, *infra,* for examples of influenza C viruses). In certain embodiments, a 3' CRS1 and a 5' CRS1 are from an influenza virus gene segment from a pandemic influenza virus. In other embodiments, a 3' CRS1 and a 5' CRS1 are from an influenza virus gene segment from a seasonal influenza virus.

[0129] In certain embodiments, a 3' CRS1 and/or a 5' CRS1 are from the same strain of the same type of influenza virus gene segment as a 3' NCR1 and/or a 5' NCR1. In other embodiments, a 3' CRS 1 and/or a 5' CRS1 are from a first strain of a type of influenza virus gene segment and a 3' NCR1 and/or a 5' NCR1 are from a different strain of the same type of influenza virus gene segment.

[0130] In certain embodiments, a 3' CRS1 comprises or consists of the 3' proximal coding region sequence of an influenza virus gene segment. The coding regions for influenza virus gene segments are known in the art or can readily be determined using standard molecular biology and virology techniques. In a specific embodiment, a 3' CRS1 comprises or consists of the 3' most 50 to 150 nucleotide, 75 to 150 nucleotides, 100 to 150 nucleotides, or 120 nucleotides of an influenza virus PB2 gene segment. In another embodiment, a 3' CRS1 comprises or consists of the 3' most 25 to 150 nucleotides, 50 to 150 nucleotides, 75 to 150 nucleotides, 100 to 150 nucleotides or 60 nucleotides of an influenza virus PB1 gene segment. In another embodiment, a 3' CRS1 comprises or consists of the 3' most 2 to 25 nucleotides, 2 to 15 nucleotides, 2 to 10 nucleotides, 5 to 150 nucleotides 25 to 150 nucleotides, 50 to 150 nucleotides, 75 to 150 nucleotides, 100 to 150 nucleotides or 9 nucleotides of an influenza virus HA gene segment. In another embodiment, a 3' CRS1 comprises or consists of the 3' most 25 to 150 nucleotides, 50 to 150 nucleotides, 75 to 150 nucleotides, 100 to 150 nucleotides or 60 nucleotides of an influenza virus NP gene segment. In another embodiment, a 3' CRS1 comprises or consists of the 3' most 25 to 250 nucleotides, 50 to 250 nucleotides, 75 to 250 nucleotides, 100 to 250 nucleotides, 125 to 250 nucleotides, 150 to 250 nucleotides, 175 to 250 nucleotides, 150 to 200 nucleotides, or 183 nucleotides of

an influenza virus NA gene segment. In another embodiment, a 3' CRS1 comprises or consists of the 3' most 25 to 250 nucleotides, 50 to 250 nucleotides, 75 to 250 nucleotides, 100 to 250 nucleotides, 125 to 250 nucleotides, 150 to 250 nucleotides, 175 to 250 nucleotides, 200 to 250 nucleotides, or 222 nucleotides of an influenza virus M gene segment. In another embodiment, a 3' CRS1 comprises or consists of the 3' most 10 to 150 nucleotides, 25 to 150 nucleotides, 50 to 150 nucleotides, 75 to 150 nucleotides, 100 to 150 nucleotides, or 35 nucleotides of an influenza virus NS gene segment. In another embodiment, a 3' CRS1 comprises or consist of the 3' most 25 to 200 nucleotides, 50 to 200 nucleotides, 50 to 150 nucleotides, 50 to 125 nucleotides, 75 to 200 nucleotides, 75 to 150 nucleotides, 100 to 200 nucleotides, 100 to 150 nucleotides, or 100 to 125 nucleotides of an influenza PA gene segment.

[0131] By way of example and not by limitation, provided in Table 5, *infra,* are examples of nucleotide sequences of a 3' proximal coding region for each segment of the influenza A/PR/8/34 (PR8) virus.

Table 5

PR8 Gene Segment	Length of Sequence	FIG./SEQ ID NO:
HA	67	FIG.4/ SEQ ID NO: 20
NA	111	FIG.6/ SEQ ID NO: 32
M	255	FIG.7/ SEQ ID NO: 38
NS	77	FIG.8/ SEQ ID NO: 44
PA	115	FIG.3/ SEQ ID NO: 14
PB1	123	FIG.2/ SEQ ID NO: 8
PB2	125	FIG.1/ SEQ ID NO: 2
NP	126	FIG.5/ SEQ ID NO: 26

[0132] Any start codon present in the 3' proximal coding region sequence of an influenza virus gene segment may be eliminated using any technique known to one of skill in the art. A start codon may be eliminated by nucleotide substitutions, deletions and/or insertions. In specific embodiments, one or more start codons present in the 3' proximal coding region of an influenza virus gene segment are eliminated by one or more nucleotide substitutions. In some embodiments, one or more start codons present in the 3' proximal coding region of an influenza virus gene segment are eliminated by one or more insertions and/or deletions. The elimination of any start codons present in the 3' proximal coding region sequence of an influenza virus gene segment should prevent the translation of the sequence.

[0133] The mRNA 5' splice site present in the 3' proximal coding region sequence of an influenza virus NS gene segment and/or the distal 5' splice site present in the 3' proximal coding region sequence of an influenza virus M gene segment may be eliminated using any technique known to one of skill in the art. Such splice sites may be eliminated by nucleotide substitutions, deletions and/or insertions. In specific emboidments, such splice sites are eliminated by nucleotide substitutions. The elimination of such splice sites such prevent unwanted alternative splicing from occurring.

[0134] In some embodiments, a 3' CRS1 comprises or consists of a nucleotide sequence that is at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to the 3' proximal coding region sequence of an influenza virus gene segment. In certain embodiments, a 3' CRS1 comprises or consists of a nucleotide sequence that is 50% to 65%, 60% to 80%, 65% to 90%, 70% to 95%, 80% to 95%, 90% to 99%, 95% to 99% identical to the 3' proximal coding region sequence of an influenza virus gene segment. In some embodiments, a 3' CRS1 comprises or consists of a nucleotide sequence that hybridizes under stringent conditions to the 3' proximal coding region sequence of an influenza virus gene segment.

[0135] In certain embodiments, a 5' CRS1 comprises or consists of the 5' proximal coding region sequence of an influenza virus gene segment. The coding regions for influenza viruses are known in the art or can readily be determined using standard molecular biology and virology techniques. In a specific embodiment, a 5' CRS1 comprises or consists of the 5' most 50 to 150 nucleotide, 75 to 150 nucleotides, 100 to 150 nucleotides, or 120 nucleotides of an influenza virus PB2 gene segment. In another embodiment, a 5' CRS1 comprises or consists of the 5' most 25 to 150 nucleotides, 50 to 150 nucleotides, 75 to 150 nucleotides, 100 to 150 nucleotides or 60 nucleotides of an influenza virus PB1 gene segment. In another embodiment, a 5' CRS1 comprises or consists of the 3' most 5 to 150 nucleotides 25 to 150 nucleotides, 50 to 150 nucleotides, 75 to 100 nucleotides, 75 to 150 nucleotides, 100 to 150 nucleotides, or 80 nucleotides of an influenza virus HA gene segment. In another embodiment, a 5' CRS1 comprises or consists of the 5' most 25 to 200 nucleotides, 50 to 200 nucleotides, 75 to 200 nucleotides, 100 to 200 nucleotides, 120 to 175 nucleotides, 120 to 150 nucleotides, or 120 nucleotides of an influenza virus NP gene segment. In another embodiment, a 5' CRS1 comprises or consists of the 5' most 25 to 250 nucleotides, 50 to 250 nucleotides, 75 to 250 nucleotides, 100 to 250 nucleotides,

125 to 250 nucleotides, 150 to 250 nucleotides, 175 to 250 nucleotides, 150 to 200 nucleotides, or 157 nucleotides of an influenza virus NA gene segment. In another embodiment, a 5' CRS1 comprises or consists of the 3' most 25 to 250 nucleotides, 50 to 250 nucleotides, 75 to 250 nucleotides, 100 to 250 nucleotides, 125 to 250 nucleotides, 150 to 250 nucleotides, 175 to 250 nucleotides, 200 to 250 nucleotides, or 220 nucleotides of an influenza virus M gene segment. In another embodiment, a 5' CRS1 comprises or consists of the 5' most 10 to 150 nucleotides, 25 to 150 nucleotides, 50 to 150 nucleotides, 75 to 150 nucleotides, 100 to 150 nucleotides, or 35 nucleotides of an influenza virus NS gene segment. In another embodiment, a 3' CRS1 comprises or consist of the 3' most 25 to 200 nucleotides, 50 to 200 nucleotides, 50 to 150 nucleotides, 50 to 125 nucleotides, 75 to 200 nucleotides, 75 to 150 nucleotides, 100 to 200 nucleotides, 100 to 150 nucleotides, or 100 to 125 nucleotides of an influenza PA gene segment.

[0136] By way of example and not by limitation, provided in Table 6, *infra,* are examples of nucleotide sequences of a 5' proximal coding region for each segment of the influenza A/PR/8/34 (PR8) virus.

Table 6

PR8 Gene Segment	Length of Sequence	FIG./ SEQ ID NO:
HA	105	FIG.4/ SEQ ID NO: 23
NA	157	FIG.6/ SEQ ID NO: 35
M	215	FIG.7/ SEQ ID NO: 41
NS	102	FIG.8/ SEQ ID NO: 47
PA	120	FIG.3/ SEQ ID NO: 17
PB1	110	FIG.2/ SEQ ID NO: 11
PB2	129	FIG.1/ SEQ ID NO: 5
NP	120	FIG.5/ SEQ ID NO: 29

[0137] In some embodiments, a 5' CRS1 comprises or consists of a nucleotide sequence that is at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99% identical to the 5' proximal coding region sequence of an influenza virus gene segment. In certain embodiments, a 5' CRS1 comprises or consists of a nucleotide sequence that is 50% to 65%, 60% to 80%, 65% to 90%, 70% to 95%, 80% to 95%, 90% to 99%, 95% to 99% identical to the 5' proximal coding region sequence of an influenza virus gene segment. In some embodiments, a 5' CRS1 comprises or consists of a nucleotide sequence that hybridizes under stringent conditions to the 5' proximal coding region sequence of an influenza virus gene segment.

5.1.3. OPEN READING FRAME OF AN INFLUENZA VIRUS GENE SEGMENT

[0138] The chimeric influenza virus gene segments of the recombinant virus provided herein comprise a mORF. A mORF comprises or consists of an open reading frame or a fragment thereof from an influenza virus gene segment or a derivative thereof, wherein the open reading frame contains one, two, three or more mutations in the influenza virus packaging signals found in the open reading frame. In a specific embodiment, a mORF comprises or consists of: either (a) at least the 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment, or an open reading frame comprising at least the 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment and a heterologous nucleotide sequence, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 of the 3' proximal nucleotides of the open reading frame of the influenza virus gene segment have been mutated; (b) at least the 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment, or an open reading frame comprising at least the 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment and a heterologous nucleotide sequence, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 of the 5' proximal nucleotides of the open reading frame of the influenza virus gene segment have been mutated; or (c) both (a) and (b). In a specific embodiment, the at least 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment and the at least 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment are from the same type of influenza virus gene segment. In other words, the at least 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment and the at least 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment are both from an HA, NA (for influenza A and B viruses), M, NS, PA, PB1, PB2, or NP influenza virus gene segment. The at least 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment and the at least 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment may be from the same type of

influenza virus gene segment (HA, NA (for influenza A and B viruses), M, NS, PA, PB1, PB2, or NP) from the same influenza virus strain. For example, the at least 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment and the at least 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment may both be from an HA influenza virus gene segment of the same influenza virus strain. Alternatively, the at least 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment and the at least 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment may be from the same type of influenza virus gene segment from two different strains of influenza virus. For example, the at least 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment and the at least 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment may be from an HA gene segment of a different influenza virus strain.

[0139] In a specific embodiment, the at least 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment and the at least 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment are from the same type of influenza virus gene segment from an influenza A virus (see Section 5.2, *infra,* for examples of influenza A viruses). In other embodiments, the at least 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment and the at least 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment are from the same type of influenza virus gene segment from an influenza B virus (see Section 5.2, *infra,* for examples of influenza B viruses). In other embodiments, the at least 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment and the at least 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment are from the same type of influenza virus gene segment from an influenza C virus (see Section *5.2, infra,* for examples of influenza C viruses). In certain embodiments, the at least 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment and the at least 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment are from the same pandemic influenza virus. In other embodiments, the at least 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment and the at least 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment are from the same seasonal influenza virus.

[0140] In one embodiment, a mORF comprises or consists of at least the 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 of the 3' proximal nucleotides of the open reading frame of the influenza virus gene segment have been mutated. In certain embodiments, a mORF comprises or consists of the 3' most 20 to 200 nucleotides, 20 to 175 nucleotides, 20 to 150 nucleotides, 20 to 125 nucleotides, 20 to 100 nucleotides, 20 to 100 nucleotides, 20 to 75 nucleotides, 20 to 50 nucleotides, or any integer in between of an open reading frame of an influenza virus gene segment, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleotides have been mutated. In a specific embodiment, a mORF comprises or consists of the 3' most 20 to 200 nucleotides, or any integer in between of an open reading frame of an influenza virus gene segment, wherein 1 to 200 nucleotides, 10 to 200 nucleotides, 20 to 200 nucleotides, 20 to 175 nucleotides, 20 to 150 nucleotides, 20 to 125 nucleotides, 20 to 100 nucleotides, 20 to 75 nucleotides, 20 to 50 nucleotides, or any integer in between have been mutated.

[0141] In one embodiment, a mORF comprises or consists of at least the 3' proximal 20 nucleotides of an open reading frame of an influenza virus gene segment and a heterologous nucleotide sequence, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 of the 3' proximal nucleotides of the open reading frame of the influenza virus gene segment have been mutated. In certain embodiments, a mORF comprises or consists of the 3' most 20 to 200 nucleotides, 20 to 175 nucleotides, 20 to 150 nucleotides, 20 to 125 nucleotides, 20 to 100 nucleotides, 20 to 100 nucleotides, 20 to 75 nucleotides, 20 to 50 nucleotides, or any integer in between of an open reading frame of an influenza virus gene segment and a heterologous nucleotide sequence, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or more nucleotides have been mutated. In a specific embodiment, a mORF comprises or consists of the 3' most 20 to 200 nucleotides, or any integer in between of an open reading frame of an influenza virus gene segment and a heterologous nucleotide sequence, wherein the 1 to 200 nucleotides, 10 to 200 nucleotides, 20 to 200 nucleotides, 20 to 200 nucleotides, 20 to 175 nucleotides, 20 to 150 nucleotides, 20 to 125 nucleotides, 20 to 100 nucleotides, 20 to 75 nucleotides, 20 to 50 nucleotides, or any integer in between have been mutated.

[0142] In another embodiment, a mORF comprises or consists of at least the 5' proximal 20 or 30 nucleotides of an open reading frame of an influenza virus gene segment, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 of the 5' proximal nucleotides of the open reading frame of the influenza virus gene segment have been mutated. In certain embodiments, a mORF comprises or consists of the 5' most 30 to 200 nucleotides, 30 to 175 nucleotides, 30 to 150 nucleotides, 30 to 125 nucleotides, 30 to 100 nucleotides, 30 to 100 nucleotides, 30 to 75 nucleotides, 230 to 50 nucleotides, or any integer in between of an open reading frame of an influenza virus gene segment, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or more nucleotides have been mutated. In a specific embodiment, a mORF comprises or consists of the 5' most 30 to 200 nucleotides, or any integer in between 30 and 200 of an open reading frame of an influenza virus gene segment, wherein the 1 to 200 nucleotides, 10 to 200 nucleotides, 20 to 200 nucleotides, 20 to 175 nucleotides, 20 to 150 nucleotides, 20 to 125 nucleotides, 20 to 100 nucleotides, 20 to 75 nucleotides, 20 to 50 nucleotides, or any integer in between have

been mutated.

[0143] In one embodiment, a mORF comprises or consists of at least the 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment and a heterologous nucleotide sequence, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 of the 5' proximal nucleotides of the open reading frame of the influenza virus gene segment have been mutated. In certain embodiments, a mORF comprises or consists of the 5' most 30 to 200 nucleotides, 30 to 175 nucleotides, 30 to 150 nucleotides, 30 to 125 nucleotides, 30 to 100 nucleotides, 30 to 100 nucleotides, 30 to 75 nucleotides, 30 to 50 nucleotides, or any integer in between of an open reading frame of an influenza virus gene segment and a heterologous nucleotide sequence, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleotides have been mutated. In a specific embodiment, a mORF comprises or consists of the 5' most 30 to 200 nucleotides, or any integer in between 30 and 200 of an open reading frame of an influenza virus gene segment and a heterologous nucleotide sequence, wherein the 1 to 200 nucleotides, 10 to 200 nucleotides, 20 to 200 nucleotides, 20 to 175 nucleotides, 20 to 150 nucleotides, 20 to 125 nucleotides, 20 to 100 nucleotides, 20 to 75 nucleotides, 20 to 50 nucleotides, or any integer in between have been mutated. In certain embodiments, when the mORF includes a heterologous nucleotide sequence, any stop codon in the open reading frame of the influenza virus gene segment is eliminated so that one open reading frame remains that allows the translation of a fusion protein.

[0144] In another embodiment, a mORF comprises or consists of at least the 3' proximal 20 nucleotides and at least the 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment, wherein at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 of the 3' proximal nucleotides and/or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 of the 5' proximal nucleotides of the open reading frame of the influenza virus gene segment have been mutated. In certain embodiments, a mORF comprises or consists of the 3' most 20 to 200 nucleotides, 20 to 175 nucleotides, 20 to 150 nucleotides, 20 to 125 nucleotides, 20 to 100 nucleotides, 20 to 100 nucleotides, 20 to 75 nucleotides, 20 to 50 nucleotides, or any integer in between of an open reading frame of an influenza virus gene segment and 5' most 30 to 200 nucleotides, 30 to 175 nucleotides, 30 to 150 nucleotides, 30 to 125 nucleotides, 30 to 100 nucleotides, 30 to 100 nucleotides, 30 to 75 nucleotides, 30 to 50 nucleotides, or any integer in between of an open reading frame of an influenza virus gene segment, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleotides in the 3' termini and/or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 or more nucleotides in the 5' termini have been mutated. In certain embodiments, when the mORF includes a heterologous nucleotide sequence, any stop codon in the open reading frame of the influenza virus gene segment is eliminated so that one open reading frame remains that allows the translation of a fusion protein.

[0145] In another embodiment, a mORF comprises or consists of (a) at least the 3' proximal 20 nucleotides and at least the 5' proximal 30 nucleotides of an open reading frame of an influenza virus gene segment, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more of the 3' proximal nucleotides and/or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 or more of the 5' proximal nucleotides of the open reading frame of the influenza virus gene segment have been mutated, and (b) a heterologous nucleotide sequence. In certain embodiments, a mORF comprises or consists of (a) the 3' most 20 to 200 nucleotides, 20 to 175 nucleotides, 20 to 150 nucleotides, 20 to 125 nucleotides, 20 to 100 nucleotides, 20 to 100 nucleotides, 20 to 75 nucleotides, 20 to 50 nucleotides, or any integer in between of an open reading frame of an influenza virus gene segment and/or 5' most 30 to 200 nucleotides, 30 to 175 nucleotides, 30 to 150 nucleotides, 30 to 125 nucleotides, 30 to 100 nucleotides, 30 to 100 nucleotides, 30 to 75 nucleotides, 30 to 50 nucleotides, or any integer in between of an open reading frame of an influenza virus gene segment, wherein 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more nucleotides in the 3' termini and/or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 or more nucleotides in the 5' termini have been mutated, and (b) a heterologous nucleotide sequence. In certain embodiments, when the mORF includes a heterologous nucleotide sequence, any stop codon in the open reading frame of the influenza virus gene segment is eliminated so that one open reading frame remains that allows the translation of a fusion protein.

[0146] In certain embodiments, a mORF comprises or consists of the entire open reading frame of an influenza virus gene segment, wherein the open reading frame of the influenza virus gene segment contains 1 to 200, 1 to 175, 1 to 150, 1 to 125, 1 to 100, 1 to 75, 1 to 50, 1 to 25, 20 to 200, 20 to 175, 20 to 150, 20 to 150, 20 to 125, 20 to 100, 20 to 75 or 20 to 50 mutations, or an integer in between. In a specific embodiments, a mORF comprises or consists of the entire open reading frame of an influenza virus gene segment, wherein the open reading frame of the influenza virus gene segment contains 1 to 200, 1 to 175, 1 to 150, 1 to 125, 1 to 100, 1 to 75, 1 to 50, 1 to 25, 20 to 200, 20 to 175, 20 to 150, 20 to 150, 20 to 125, 20 to 100, 20 to 75 or 20 to 50 mutations (or an integer in between) at the 3' termini and/or 1 to 200, 1 to 175, 1 to 150, 1 to 125, 1 to 100, 1 to 75, 1 to 50, 1 to 25, 20 to 200, 20 to 175, 20 to 150, 20 to 150, 20 to 125, 20 to 100, 20 to 75 or 20 to 50 mutations (or an integer in between) at the 5' termini. For example, a mORF may comprise or consist of the entire open reading frame of the influenza virus gene segment HA, NA, PA, PB1, PB2, NP, NS or M, wherein the open reading frame of the influenza virus gene segment contains 1 to 200, 1 to 175, 1

to 150, 1 to 125, 1 to 100, 1 to 75, 1 to 50, or 1 to 25 mutations (or an integer in between) at the 3' termini and/or 1 to 200, 1 to 175, 1 to 150, 1 to 125, 1 to 100, 1 to 75, 1 to 50, or 1 to 25 mutations (or an integer in between) at the 5' termini. In certain embodiments, a mORF comprises or consists of the entire open reading frame of an influenza virus gene segment, wherein the open reading frame of the influenza virus gene segment contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 mutations at the 3' termini and/or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 mutations at the 5' termini.

[0147] In a specific embodiments, a mORF comprises or consists of the entire open reading frame of an influenza virus gene segment and a heterologous nucleotide sequence, wherein the open reading frame of the influenza virus gene segment contains 1 to 200, 1 to 175, 1 to 150, 1 to 125, 1 to 100, 1 to 75, 1 to 50, or 1 to 25 mutations (or an integer in between) at the 3' termini and/or 1 to 200, 1 to 175, 1 to 150, 1 to 125, 1 to 100, 1 to 75, 1 to 50, or 1 to 25 mutations (or an integer in between) at the 5' termini. In certain embodiments, a mORF comprises or consists of the entire open reading frame of an influenza virus gene segment and a heterolgous nucleotide sequence, wherein the open reading frame of the influenza virus gene segment contains 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 mutations at the 3' termini and/or 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 mutations at the 5' termini. In certain embodiments, when the mORF includes a heterologous nucleotide sequence, any stop codon in the open reading frame of the influenza virus gene segment is eliminated so that one open reading frame remains that allows the translation of a fusion protein.

[0148] The open reading frames of influenza virus gene segments are known in the art or can readily be determined using standard molecular biology and virology techniques. For example and not by limitation, the open reading frames for each gene product of influenza WSN virus is provided below in Table 7, *infra.*

Table 7

WSN ORF	Length of Sequence	FIG./SEQ ID NO:
HA	1698	FIG. 17/ SEQ ID NO: 182
NA	1362	FIG. 18/ SEQ ID NO: 86
M1/M2	759/294	FIG. 19/ SEQ ID NO: 90
NS1/NS2	693/366	FIG. 20/ SEQ ID NO: 84
PA	2151	FIG. 21/ SEQ ID NO: 98
PB1	2274	FIG. 22/ SEQ ID NO: 102
PB2	2280	FIG. 23/ SEQ ID NO: 106
NP	1497	FIG. 24/ SEQ ID NO: 110

[0149] In specific embodiments, mutations in the open reading frame of an influenza virus gene segment or a fragment thereof mutate or eliminate one or more or all of the packaging signals found in the open reading frame. In particular embodiments, such packaging signals are found in the 3' and 5' ends of the sequence. According to the invention mutations in the open reading frame of an influenza virus gene segment or a fragment thereof are silent mutations, *i.e.,* mutations that alter the nucleotide sequence of the open reading frame but do not alter the amino acid sequence encoded by the open reading frame. Most naturally occurring amino acids are encoded by multiple different codons (methionine and tryptophan are the exception)--a phenomenon that has been termed degeneracy of the genetic code. Thus, certain mutations of a codon can result in a different nucleotide sequence while encoding the same amino acid.

[0150] In certain embodiments, mutations in the open reading frame of an influenza virus gene segment or a fragment thereof result in a conservative amino acid exchange in the protein, *i.e.,* a mutation that results in an amino acid exchange where the new amino acid has very similar chemical properties as the original, wild type amino acid. Such conservative amino acid exchanges include amino acid exchanges such as acidic amino acid for acidic amino acid; basic amino acid for basic amino acid; aliphatic amino acid for aliphatic amino acid; and aromatic amino acid for aromatic amino acid.

[0151] By way of example and not by limitation, examples of silent mutations that may be introduced into the open reading frame of each gene segment of the influenza PR8 virus are provided below in Table 8, *infra.*

Table 8

PR8 Gene Segment	Wild-Type - 3' Termini (FIG.; SEQ ID NO:)	Mutated - 3' Termini (FIG.; SEQ ID NO:)	Wild-Type - 5' Termini (FIG.; SEQ ID NO:)	Mutated - 5' Termini (FIG.; SEQ ID NO:)
HA	FIG. 12A; SEQ ID NO: 61	FIG. 12B: SEQ ID NO: 62	FIG. 12C; SEQ ID NO: 63	FIG. 12D: SEQ ID NO: 64
NA	FIG. 14A; SEQ ID NO: 69	FIG. 14B; SEQ ID NO: 70	FIG. 14C; SEQ ID NO: 71	FIG. 14D; SEQ ID NO: 72
M	FIG. 15A; SEQ ID NO: 73	FIG. 15B; SEQ ID NO: 74	FIG. 15C; SEQ ID NO: 75	FIG. 15D; SEQ ID NO: 76
NS	FIG. 16A; SEQ ID NO: 77	FIG. 16B; SEQ ID NO: 78	FIG. 16C; SEQ ID NO: 79	FIG. 16D; SEQ ID NO: 80
PA	FIG. 11A; SEQ ID NO: 57	FIG. 11B; SEQ ID NO: 58	FIG. 11C; SEQ ID NO: 59	FIG. 11D; SEQ ID NO: 60
PB1	FIG. 10A; SEQ ID NO: 53	FIG. 10B; SEQ ID NO: 54	FIG. 10C; SEQ ID NO: 55	FIG. 10D; SEQ ID NO: 56
PB2	FIG. 9A; SEQ ID NO: 49	FIG. 9B; SEQ ID NO: 50	FIG. 9C; SEQ ID NO: 51	FIG. 9D; SEQ ID NO: 52
NP	FIG. 13A; SEQ ID NO: 65	FIG. 13B; SEQ ID NO: 66	FIG. 13C; SEQ ID NO: 67	FIG. 13D; SEQ ID NO: 68

[0152] In certain embodiments, a mORF may include a heterologous nucleotide sequence. The heterologous nucleotide sequence is generally in frame with the open reading frame of an influenza virus gene segment or a derivative or a fragment thereof. In a specific embodiment, the heterologous nucleotide sequence encodes an antigen of any infectious pathogen or associated with any disease that is capable of eliciting an immune response. In a specific embodiment, the antigen is a glycoprotein. In certain embodiments, the heterologous nucleotide sequence encodes a viral antigen. In other embodiments, the heterologous nucleotide sequence encodes a bacterial antigen *(e.g.,* bacterial coat protein). In other embodiments, the heterologous nucleotide sequence encodes parasitic antigen *(e.g.,* a protozoan antigen). In another embodiment, the heterologous nucleotide sequence encodes a fungal antigen.

[0153] In some embodiments, the heterologous nucleotide sequence encodes a tumor antigen or tumor associated antigen. In some embodiments, the heterologous nucleotide sequence encodes a cytokine or growth factor. In certain embodiments, the heterologous nucleotide sequence encodes a peptide tag, such as flag tag. In some embodiments, the heterologous nucleotide sequence encodes a detectable substance.

[0154] Non-limiting examples of viral antigens include antigens from adenoviridae *(e.g.,* mastadenovirus and aviadenovirus), herpesviridae *(e.g.,* herpes simplex virus 1, herpes simplex virus 2, herpes simplex virus 5, herpes simplex virus 6, Epstein-Barr virus, HHV6-HHV8 and cytomegalovirus), leviviridae *(e.g.,* levivirus, enterobacteria phase MS2, allolevirus), poxviridae *(e.g.,* chordopoxvirinae, parapoxvirus, avipoxvirus, capripoxvirus, leporiipoxvirus, suipoxvirus, molluscipoxvirus, and entomopoxvirinae), papovaviridae *(e.g.,* polyomavirus and papillomavirus), paramyxoviridae (e.g., paramyxovirus, parainfluenza virus 1, mobillivirus *(e.g.,* measles virus), rubulavirus *(e.g.,* mumps virus), pneumonovirinae *(e.g.,* pneumovirus, human respiratory synctial virus), human respiratory syncytial virus and metapneumovirus *(e.g.,* avian pneumovirus and human metapneumovirus)), picornaviridae *(e.g.,* enterovirus, rhinovirus, hepatovirus *(e.g.,* human hepatits A virus), cardiovirus, and apthovirus), reoviridae *(e.g.,* orthoreovirus, orbivirus, rotavirus, cypovirus, fijivirus, phytoreovirus, and oryzavirus), retroviridae *(e.g.,* mammalian type B retroviruses, mammalian type C retroviruses, avian type C retroviruses, type D retrovirus group, BLV-HTLV retroviruses, lentivirus *(e.g.* human immunodeficiency virus 1 and human immunodeficiency virus 2 *(e.g.,* HIV gp160), spumavirus), flaviviridae *(e.g.,* hepatitis C virus, dengue virus, West Nile virus), hepadnaviridae *(e.g.,* hepatitis B virus), togaviridae *(e.g.,* alphavirus *(e.g.,* sindbis virus) and rubivirus *(e.g.,* rubella virus)), rhabdoviridae *(e.g.,* vesiculovirus, lyssavirus, ephemerovirus, cytorhabdovirus, and necleorhabdovirus), arenaviridae *(e.g.,* arenavirus, lymphocytic choriomeningitis virus, Ippy virus, and lassa virus), and coronaviridae *(e.g.,* coronavirus and torovirus). In a specific embodiment, the viral antigen is HIV gp120, HIV nef, RSV F glycoprotein, RSV G glycoprotein, HTLV tax, herpes simplex virus glycoprotein *(e.g.,* gB, gC, gD, and gE) or hepatitis B surface antigen, hepatitis C virus E protein or coronavirus spike protein.

[0155] Non-limiting examples of bacterial antigens include antigens from bacteria of the *Aquaspirillum* family, *Azospirillum* family, *Azotobacteraceae* family, *Bacteroidaceae* family, *Bartonella* species, *Bdellovibrio* family, *Campylobacter*

species, *Chlamydia* species *(e.g., Chlamydia pneumoniae), clostridium, Enterobacteriaceae* family *(e.g., Citrobacter* species, *Edwardsiella, Enterobacter aerogenes, Erwinia* species, *Escherichia coli, Hafnia* species, *Klebsiella* species, *Morganella* species, *Proteus vulgaris, Providencia, Salmonella* species, *Serratia marcescens,* and *Shigella flexneri), Gardinella* family, *Haemophilus influenzae, Halobacteriaceae* family, *Helicobacter* family, *Legionallaceae* family, *Listeria* species, *Methylococcaceae* family, *mycobacteria (e.g., Mycobacterium tuberculosis), Neisseriaceae* family, *Oceanospirillum* family, *Pasteurellaceae* family, *Pneumococcus* species, *Pseudomonas* species, *Rhizobiaceae* family, *Spirillum* family, *Spirosomaceae* family, *Staphylococcus (e.g.,* methicillin resistant *Staphylococcus aureus* and *Staphylococcus pyrogenes), Streptococcus (e.g., Streptococcus enteritidis, Streptococcus fasciae,* and *Streptococcus pneumoniae), Helicobacter* family, *Yersinia* family, *Bacillus antracis* and *Vampirovibrio* family.

[0156] Non-limiting examples of parasite antigens include antigens from a parasite such as an amoeba, a malarial parasite, Plasmodium, Trypanosoma cruzi. Non-limiting examples of fungal antigens include antigens from fungus of Absidia species *(e.g.,* Absidia corymbifera and Absidia ramosa), Aspergillus species, *(e.g.,* Aspergillus flavus, Aspergillus fumigatus, Aspergillus nidulans, Aspergillus niger, and Aspergillus terreus), Basidiobolus ranarum, Blastomyces dermatitidis, Candida species *(e.g.,* Candida albicans, Candida glabrata, Candida kerr, Candida krusei, Candida parapsilosis, Candida pseudotropicalis, Candida quillermondii, Candida rugosa, Candida stellatoidea, and Candida tropicalis), Coccidioides immitis, Conidiobolus species, Cryptococcus neoforms, Cunninghamella species, dermatophytes, Histoplasma capsulatum, Microsporum gypseum, Mucor pusillus, Paracoccidioides brasiliensis, Pseudallescheria boydii, Rhinosporidium seeberi, Pneumocystis carinii, Rhizopus species *(e.g.,* Rhizopus arrhizus, Rhizopus oryzae, and Rhizopus microsporus), Saccharomyces species, Sporothrix schenckii, zygomycetes, and classes such as Zygomycetes, Ascomycetes, the Basidiomycetes, Deuteromycetes, and Oomycetes.

[0157] Non-limiting examples of tumor associated antigens include MAGE-1, MAGE-3, BAGE, GAGE-1, GAGE-2, N-acetylglucosaminyltransferase-V, p-15, MART-1/MelanA, TRP-1 (gp75), Tyrosinase, cyclin-dependent kinase 4, MUM-1, CDK4, HER-2/neu, human papillomavirus-E6, human papillomavirus E7 , MUC-1, caspase-8, CD5, CD20, CEA, mucin-1, Lewisx, CA-125, epidermal growth factor receptor, p185HER2, IL-2R, tenascin, antigens associated with a metalloproteinase, and CAMPATH-1. Non-limiting examples of cytokines and growth factors include interleukin (IL)-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12, IL-15, IL-18, IL-22, IFN-alpha, IFN-beta, and IFN-beta. Non-limiting examples of detectable substances include various enzymes, such as, but not limited to, horseradish peroxidase, alkaline phosphatase, beta-galactosidase, or acetylcholinesterase; prosthetic groups, such as, but not limited to, streptavidin/biotin and avidin/biotin; and bioluminescent materials, such as but not limited to, luciferase, luciferin, and aequorin.

[0158] In specific embodiments, the heterologous nucleotide sequence encodes a respiratory pathogen antigen. Non-limiting examples of respiratory virus antigens include the F, G, or M2 protein of RSV, the spike protein of a Coronavirus (e.g., SARS, HuCoV), the F protein of human metapneumovirus, the F or HN protein of parainfluenza virus, the G or F protein of Hendra virus, the G or F protein of Nipah virus, or the capsid protein of Adenovirus. In a specific embodiment, the respiratory virus antigen is an influenza virus antigen from a different type, subtype, or strain of influenza virus.

5.2 INFLUENZA VIRUS COMPRISING CHIMERIC INFLUENZA VIRUS GENE SEGMENTS

[0159] In one aspect, provided herein are recombinant influenza viruses as defined in the claims comprising two, three, four, five, six, seven or eight chimeric influenza virus gene segments described herein. In a specific embodiment, provided herein are recombinant influenza viruses comprising two or more chimeric influenza virus gene segments described herein, wherein the two or more chimeric influenza virus gene segments cosegregate (otherwise referred to herein as a "cosegregating chimeric influenza virus gene segments"). A group of cosegregating chimeric influenza virus gene segments may include two, three, four, five, six, seven or eight chimeric influenza virus gene segments. In certain embodiments, two or more chimeric influenza virus gene segments cosegregate at least 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% of the time as determined by a technique known to one of skill in the art. In some embodiments, two or more chimeric influenza virus gene segments cosegregate 10% to 50%, 10% to 75%, 10% to 95%, 10% to 99.5%, 25% to 50%, 25% to 75%, 25% to 99.5%, 50% to 75%, 50% to 99.5%, 75% to 99.5, 80% to 99.5%, 90% to 99.5%, or 95% to 99.5% of the time as determined by a technique known to one of skill in the art. One example of such a technique may comprise coinfecting the cells with a wild-type virus and a recombinant influenza virus described herein, picking single plaques, and determining the genomic composition of each plaque. Without being bound by theory, the chimeric influenza virus gene segments have a reduced the ability to reassort independently of each other with other influenza virus gene segments, and thus, the reassortment of the recombinant influenza virus with other influenza viruses *(e.g.,* wild-type influenza viruses) is reduced or inhibited. In some embodiments, the reassortment of the recombinant influenza virus with other influenza viruses is less than 40%, 35%, 30%, 25%, 20%, 15%, 10% or 5% as determined by the percentage of viral plaques containing reassorted influenza viruses with one or more chimeric influenza virus gene segments that have reassorted independently from one or more other chimeric influenza virus gene segments. Recombinant influenza viruses that are unable to reassort will produce fewer viral plaques that contain viruses with one or more chimeric influenza virus gene segments that has reasserted

independently of one or more other chimeric influenza virus gene segments.

[0160] In certain embodiments, a recombinant influenza virus provided herein comprises two chimeric influenza virus gene segments that cosegregate. The first and second chimeric influenza virus gene segments contain packaging signals obtained or derived from a first and a second type of influenza virus gene segment as provided, *e.g.,* in Table 9, *infra.*

Table 9

	3' NCR1 & 5' NCR1	3' CRS1 &/or 5' CRS1	mORF
First Chimeric Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment
Second Chimeric Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment

The first and second types of influenza virus gene segments refer to two different influenza virus gene segments. For example, the first type of influenza virus gene segment may be an HA influenza virus gene segment and the second type of influenza virus gene segment may be an NS influenza virus gene segment.

[0161] In specific embodiments, a recombinant influenza virus may comprise a first and a second chimeric influenza virus gene segment, wherein:

(a) the first chimeric influenza virus gene segment comprises:

(i) a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof;
(ii) a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the first type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;
(iii) an open reading frame of a second influenza virus gene segment, or an open reading frame comprising an open reading frame of a second type of influenza virus gene segment or a fragment thereof and a heterologous nucleotide sequence, wherein 3' and 5' proximal nucleotides of the open reading frame of the second type of influenza virus gene segment have been mutated;
(iv) a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the first type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and
(v) a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof; and wherein

(b) the second chimeric influenza virus gene segment comprises:

(i) a 3' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of the second type of influenza virus gene segment or a fragment thereof;
(ii) a 3' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%,

at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the second type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;

(iii) an open reading frame of the first type of influenza virus gene segment, or an open reading frame comprising an open reading frame of the first type of influenza virus gene segment and a heterologous nucleotide sequence, wherein 3' and 5' proximal nucleotides of the open reading frame of the first type of influenza virus gene segment have been mutated;

(iv) a 5' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the second type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and

(v) a 5' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the second type of influenza virus gene segment or a fragment thereof.

In certain embodiments, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site

[0162] In certain embodiments, a recombinant influenza virus provided herein comprises three chimeric influenza virus gene segments that cosegregate. The first, second and third chimeric influenza virus gene segments contain packaging signals obtained or derived from a first, a second and a third type of influenza virus gene segment as provided, *e.g.,* in Table 10, *infra.*

Table 10

	3' NCR1 & 5' NCR1	**3' CRS1 &/or 5' CRS1**	**mORF**
First Chimeric Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment
Second Chimeric Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment
Third Chimeric Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment

The first, second and third types of influenza virus gene segments refer to three different influenza virus gene segments. For example, the first type of influenza virus gene segment may be an HA influenza virus gene segment, the second type of influenza virus gene segment may be an NS influenza virus gene segment, and the third type of influenza virus gene segment may be an NP influenza virus gene segment.

[0163] In specific embodiments, a recombinant influenza virus may comprise a first, a second, and a third chimeric influenza virus gene segment, wherein:

(a) the first chimeric influenza virus gene segment comprises:

(i) a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of a first type of influenza virus gene segment or a fragment thereof;

(ii) a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%,

at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the first type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;

(iii) an open reading frame of a third type of influenza virus gene segment, or an open reading frame comprising an open reading frame of a third type of influenza virus gene segment or a fragment thereof and a heterologous nucleotide sequence, wherein 3' and 5' proximal nucleotides of the open reading frame of the third type of influenza virus gene segment have been mutated;

(iv) a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the first type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the first type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and

(v) a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the first type of influenza virus gene segment or a fragment thereof; and wherein

(b) the second chimeric influenza virus gene segment comprises:

(i) a 3' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of the second type of influenza virus gene segment or a fragment thereof;

(ii) a 3' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the second type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;

(iii) an open reading frame of the first type of influenza virus gene segment, or an open reading frame comprising an open reading frame of the first type of influenza virus gene segment or a fragment thereof and a heterologous nucleotide sequence, wherein 3' and 5' proximal nucleotides of the open reading frame of the first type of influenza virus gene segment have been mutated;

(iv) a 5' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the second type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the second type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and

(v) a 5' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the second type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the second type of influenza virus gene segment or a fragment thereof; wherein

(c) the third chimeric influenza virus gene segment comprises:

(i) a 3' NCR of the third type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' NCR of the third type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 3' NCR of the third type of influenza virus gene segment or a fragment thereof;

(ii) a 3' proximal coding region sequence of the third type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%,

at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 3' proximal coding region sequence of the third type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 3' proximal coding region sequence of the third type of influenza virus gene segment, wherein any start codon present in the sequence in (ii) has been eliminated;

(iii) an open reading frame of the second type of influenza virus gene segment, or an open reading frame comprising an open reading frame of the second type of influenza virus gene segment or a fragment thereof and a heterologous nucleotide sequence, wherein 3' and 5' proximal nucleotides of the open reading frame of the second type of influenza virus gene segment have been mutated;

(iv) a 5' proximal coding region sequence of the third type of influenza virus gene segment, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' proximal coding region sequence of the third type of influenza virus gene segment, or a nucleotide sequence that hybridizes under stringent conditions to a 5' proximal coding region sequence of the third type of influenza virus gene segment, wherein the sequence in (iv) is not translated; and

(v) a 5' NCR of the third type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that is at least 50% (in some embodiments, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 98%) identical to a 5' NCR of the third type of influenza virus gene segment or a fragment thereof, or a nucleotide sequence that hybridizes under stringent conditions to a 5' NCR of the third type of influenza virus gene segment or a fragment thereof.

In certain embodiments, the 3' proximal coding region sequence is derived from an influenza virus NS or M gene segment. In a specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus NS gene segment and the 3' proximal coding region has been mutated so as to eliminate the mRNA 5' splice site. In another specific embodiment, the 3' proximal coding region sequence is derived from an influenza virus M gene segment and the 3' proximal coding region has been mutated so as to eliminate the distal 5' splice site.

[0164] In certain embodiments, a recombinant influenza virus provided herein comprises four chimeric influenza virus gene segments that cosegregate. The first, second, third and fourth chimeric influenza virus gene segments contain packaging signals obtained or derived from a first, a second, a third and a fourth type of influenza virus gene segment as provided, *e.g.,* in Table 11, *infra.*

Table 11

	3' NCR1 & 5' NCR1	**3' CRS1 &/or 5' CRS1**	**mORF**
First Chimeric Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	4rd Type of Influenza Virus Gene Segment
Second Chimeric Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment
Third Chimeric	3rd Type of Influenza	3rd Type of Influenza	2nd Type of Influenza
Influenza Virus Gene Segment	Virus Gene Segment	Virus Gene Segment	Virus Gene Segment
Fourth Chimeric Influenza Virus Gene Segment	4th Type of Influenza Virus Gene Segment	4th Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment

The first, second, third and fourth types of influenza virus gene segments refer to four different influenza virus gene segments. For example, the first type of influenza virus gene segment may be an HA influenza virus gene segment, the second type of influenza virus gene segment may be an NS influenza virus gene segment, the third type of influenza virus gene segment may be an NP influenza virus gene segment, and the fourth type of influenza virus gene segment may be an PB1.

[0165] In certain embodiments, a recombinant influenza virus provided herein comprises five chimeric influenza virus gene segments that cosegregate. The first, second, third, fourth and fifth chimeric influenza virus gene segments contain packaging signals obtained or derived from a first, a second, a third, a fourth and a fifth type of influenza virus gene segment as provided, *e.g.,* in Table 12, *infra.*

Table 12

	3' NCR1 & 5' NCR1	3' CRS1 &/or 5' CRS1	mORF
First Chimeric Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment
Second Chimeric Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment
Third Chimeric Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment
Fourth Chimeric Influenza Virus Gene Segment	5th Type of Influenza Virus Gene Segment	5th Type of Influenza Virus Gene Segment	4th Type of Influenza Virus Gene Segment
Fifth Chimeric	4th Type of Influenza	4th Type of Influenza	5th Type of Influenza
Influenza Virus Gene Segment	Virus Gene Segment	Virus Gene Segment	Virus Gene Segment

The first, second, third, fourth and fifth types of influenza virus gene segments refer to five different influenza virus gene segments. For example, the first type of influenza virus gene segment may be an HA influenza virus gene segment, the second type of influenza virus gene segment may be an NS influenza virus gene segment, the third type of influenza virus gene segment may be an NP influenza virus gene segment, the fourth type of influenza virus gene segment may be a PB1 influenza virus gene segment, and the fifth type of influenza virus gene segment may be a PB2 influenza virus gene segment.

[0166] In certain embodiments, a recombinant influenza virus provided herein comprises six chimeric influenza virus gene segments that cosegregate. The first, second, third, fourth, fifth and sixth chimeric influenza virus gene segments contain packaging signals obtained or derived from a first, a second, a third, a fourth, a fifth and a sixth type of influenza virus gene segment as provided, *e.g.,* in Table 13, *infra.*

Table 13

	3' NCR1 & 5' NCR1	3' CRS1 &/or 5' CRS1	mORF
First Chimeric Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment
Second Chimeric Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment
Third Chimeric Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment	4th Type of Influenza Virus Gene Segment
Fourth Chimeric Influenza Virus Gene Segment	4th Type of Influenza Virus Gene Segment	4th Type of Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment
Fifth Chimeric Influenza Virus Gene	5th Type of Influenza Virus Gene Segment	5th Type of Influenza Virus Gene Segment	6th Type of Influenza Virus Gene Segment
Segment			
Sixth Chimeric Influenza Virus Gene Segment	6th Type of Influenza Virus Gene Segment	6th Type of Influenza Virus Gene Segment	5th Type of Influenza Virus Gene Segment

The first, second, third, fourth, fifth and sixth types of influenza virus gene segments refer to six different influenza virus gene segments. For example, the first type of influenza virus gene segment may be an HA influenza virus gene segment, the second type of influenza virus gene segment may be an NS influenza virus gene segment, the third type of influenza virus gene segment may be an NP influenza virus gene segment, the fourth type of influenza virus gene segment may be a PB 1 influenza virus gene segment, the fifth type of influenza virus gene segment may be a PB2 influenza virus gene segment, and the sixth type of influenza virus gene segment from a PA influenza virus gene segment.

[0167] In certain embodiments, a recombinant influenza virus provided herein comprises seven chimeric influenza virus gene segments that cosegregate. The first, second, third, fourth, fifth, sixth and seventh chimeric influenza virus

gene segments contain packaging signals obtained or derived from a first, a second, a third, a fourth, a fifth, a sixth and a seventh type of influenza virus gene segment as provided, *e.g.,* in Table 14, *infra.*

Table 14

	3' NCR1 & 5' NCR1	3' CRS1 &/or 5' CRS1	mORF
First Chimeric Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment
Second Chimeric Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment
Third Chimeric Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment	4th Type of Influenza Virus Gene Segment
Fourth Chimeric Influenza Virus Gene	4th Type of Influenza Virus Gene Segment	4th Type of Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment
Segment			
Fifth Chimeric Influenza Virus Gene Segment	5th Type of Influenza Virus Gene Segment	5th Type of Influenza Virus Gene Segment	6th Type of Influenza Virus Gene Segment
Sixth Chimeric Influenza Virus Gene Segment	6th Type of Influenza Virus Gene Segment	6th Type of Influenza Virus Gene Segment	7th Type of Influenza Virus Gene Segment
Seventh Chimeric Influenza Virus Gene Segment	7th Type of Influenza Virus Gene Segment	7th Type of Influenza Virus Gene Segment	5th Type of Influenza Virus Gene Segment

The first, second, third, fourth, fifth, sixth and seventh types of influenza virus gene segments refer to seven different influenza virus gene segments. For example, the first type of influenza virus gene segment may be an HA influenza virus gene segment, the second type of influenza virus gene segment may be an NS influenza virus gene segment, the third type of influenza virus gene segment may be an NP influenza virus gene segment, the fourth type of influenza virus gene segment may be a PB1 influenza virus gene segment, the fifth type of influenza virus gene segment may be a PB2 influenza virus gene segment, the sixth type of influenza virus gene segment from a PA influenza virus gene segment, and a seventh type of influenza virus gene segment from an M influenza virus gene segment.

[0168] In certain embodiments, a recombinant influenza virus provided herein comprises eight chimeric influenza virus gene segments that cosegregate. The first, second, third, fourth, fifth, sixth, seventh and eight chimeric influenza virus gene segments contain packaging signals obtained or derived from a first, a second, a third, a fourth, a fifth, a sixth, a seventh and an eighth type of influenza virus gene segment as provided, e.g., in Table 15, *infra.*

Table 15

	3' NCR1 & 5' NCR1	3' CRS1 &/or 5' CRS1	mORF
First Chimeric Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment
Second Chimeric Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	2nd Type of Influenza Virus Gene Segment	1st Type of Influenza Virus Gene Segment
Third Chimeric Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment	4th Type of Influenza Virus Gene Segment
Fourth Chimeric Influenza Virus Gene Segment	4th Type of Influenza Virus Gene Segment	4th Type of Influenza Virus Gene Segment	3rd Type of Influenza Virus Gene Segment
Fifth Chimeric Influenza Virus Gene Segment	5th Type of Influenza Virus Gene Segment	5th Type of Influenza Virus Gene Segment	6th Type of Influenza Virus Gene Segment
Sixth Chimeric Influenza Virus Gene Segment	6th Type of Influenza Virus Gene Segment	6th Type of Influenza Virus Gene Segment	5th Type of Influenza Virus Gene Segment

(continued)

	3' NCR1 & 5' NCR1	3' CRS1 &/or 5' CRS1	mORF
Seventh Chimeric Influenza Virus Gene Segment	7th Type of Influenza Virus Gene Segment	7th Type of Influenza Virus Gene Segment	8th Type of Influenza Virus Gene Segment
Eighth Chimeric Influenza Virus Gene Segment	8th Type of Influenza Virus Gene Segment	8th Type of Influenza Virus Gene Segment	7th Type of Influenza Virus Gene Segment

The first, second, third, fourth, fifth, sixth, seventh and eighth types of influenza virus gene segments refer to eight different influenza virus gene segments. For example, the first type of influenza virus gene segment may be an HA influenza virus gene segment, the second type of influenza virus gene segment may be an NS influenza virus gene segment, the third type of influenza virus gene segment may be an NP influenza virus gene segment, the fourth type of influenza virus gene segment may be a PB1 influenza virus gene segment, the fifth type of influenza virus gene segment may be a PB2 influenza virus gene segment, the sixth type of influenza virus gene segment from a PA influenza virus gene segment, a seventh type of influenza virus gene segment from an M influenza virus gene segment, and an eighth type of influenza virus gene segment from a neuraminidase (NA) influenza virus gene segment.

[0169] In certain embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from the same type of influenza virus, the same subtype of influenza virus, or the same strain of influenza virus. In certain embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a mORF, a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from the same type of influenza virus, the same subtype of influenza virus, or the same strain of influenza virus.

[0170] In some embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1 and a 5' NCR1 from one type of influenza virus, one subtype of influenza virus, or one influenza virus strain and a 3' CRS 1 and a 5'CRS1 from a different type of influenza virus, a different subtype of influenza virus, or a different strain of influenza virus. In some embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from one type of influenza virus, one subtype of influenza virus, or one influenza virus strain and a mORF from a different type of influenza virus, a different subtype of influenza virus, or a different strain of influenza virus.

[0171] In some embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from a pandemic influenza virus and a mORF from a seasonal influenza virus. In other embodiments, a recombinant influenza virus comprises a chimeric influenza gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from a seasonal influenza virus and a mORF from a pandemic influenza virus. In certain embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a mORF from a seasonal or pandemic influenza virus.

[0172] In certain embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from an influenza A virus. In some embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1, a 5'CRS1 and a mORF from an influenza A virus. In specific embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from the same subtype or strain of influenza A virus. In certain embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a mORF, a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from the same subtype or strain of influenza A virus. In some embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1 and a 5' NCR1 from one subtype of influenza A virus or one influenza A virus strain and a 3' CRS1 and a 5'CRS1 from a different subtype of influenza A virus or a different strain of influenza A virus. In some embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from one subtype of influenza A virus or one influenza A virus strain and a mORF from a different subtype of influenza A virus or a different strain of influenza A virus.

[0173] Non-limiting examples of influenza A viruses include subtype H10N4, subtype H10N5, subtype H10N7, subtype H10N8, subtype H10N9, subtype H11N1, subtype H11N13, subtype H11N2, subtype H11N4, subtype H11N6, subtype H11N8, subtype H11N9, subtype H12N1, subtype H12N4, subtype H12N5, subtype H12N8, subtype H13N2, subtype H13N3, subtype H13N6, subtype H13N7, subtype H14N5, subtype H14N6, subtype H15N8, subtype H15N9, subtype H16N3, subtype H1N1, subtype H1N2, subtype H1N3, subtype H1N6, subtype H1N9, subtype H2N1, subtype H2N2, subtype H2N3, subtype H2N5, subtype H2N7, subtype H2N8, subtype H2N9, subtype H3N1, subtype H3N2, subtype H3N3, subtype H3N4, subtype H3N5, subtype H3N6, subtype H3N8, subtype H3N9, subtype H4N1, subtype H4N2, subtype H4N3, subtype H4N4, subtype H4N5, subtype H4N6, subtype H4N8, subtype H4N9, subtype H5N1, subtype HSN2, subtype HSN3, subtype HSN4, subtype HSN6, subtype HSN7, subtype HSN8, subtype HSN9, subtype H6N1,

subtype H6N2, subtype H6N3, subtype H6N4, subtype H6N5, subtype H6N6, subtype H6N7, subtype H6N8, subtype H6N9, subtype H7N1, subtype H7N2, subtype H7N3, subtype H7N4, subtype H7N5, subtype H7N7, subtype H7N8, subtype H7N9, subtype H8N4, subtype H8N5, subtype H9N1, subtype H9N2, subtype H9N3, subtype H9N5, subtype H9N6, subtype H9N7, subtype H9N8, and subtype H9N9.

[0174] Specific examples of strains of influenza A virus include, but are not limited to: A/sw/Iowa/15/30 (H1N1); A/WSN/33 (H1N1); A/eq/Prague/1/56 (H7N7); A/PR/8/34; A/mallard/Potsdam/178-4/83 (H2N2); A/herring gull/DE/712/88 (H16N3); A/sw/Hong Kong/168/1993 (H1N1); A/mallard/Alberta/211/98 (H1N1); A/shorebird/Delaware/168/06 (H16N3); A/sw/Netherlands/25/80 (H1N1); A/sw/Germany/2/81 (H1N1); A/sw/Hannover/1/81 (H1N1); A/sw/Potsdam/1/81 (H1N1); A/sw/Potsdam/15/81 (H1N1); A/sw/Potsdam/268/81 (H1N1); A/sw/Finistere/2899/82 (H1N1); A/sw/Potsdam/35/82 (H3N2); A/sw/Cote d'Armor/3633/84 (H3N2); A/sw/Gent/1/84 (H3N2); A/sw/Netherlands/12/85 (H1N1); A/sw/Karrenzien/2/87 (H3N2); A/sw/Schwerin/103/89 (H1N1); A/turkey/Germany/3/91 (H1N1); A/sw/Germany/8533/91 (H1N1); A/sw/Belgium/220/92 (H3N2); A/sw/Gent/V230/92 (H1N1); A/sw/Leipzig/145/92 (H3N2); A/sw/Re220/92hp (H3N2); A/sw/Bakum/909/93 (H3N2); A/sw/Schleswig-Holstein/1/93 (H1N1); A/sw/Scotland/419440/94 (H1N2); A/sw/Bakum/5/95 (H1N1); A/sw/Best/5C/96 (H1N1); A/sw/England/17394/96 (H1N2); A/sw/Jena/5/96 (H3N2); A/sw/Oedenrode/7C/96 (H3N2); A/sw/Lohne/1/97 (H3N2); A/sw/Cote d'Armor/790/97 (H1N2); A/sw/Bakum/1362/98 (H3N2); A/sw/Italy/1521/98 (H1N2); A/sw/Italy/1553-2/98 (H3N2); A/sw/Italy/1566/98 (H1N1); A/sw/Italy/1589/98 (H1N1); A/sw/Bakum/8602/99 (H3N2); A/sw/Cotes d'Armor/604/99 (H1N2); A/sw/Cote d'Armor/1482/99 (H1N1); A/sw/Gent/7625/99 (H1N2); A/Hong Kong/1774/99 (H3N2); A/sw/Hong Kong/5190/99 (H3N2); A/sw/Hong Kong/5200/99 (H3N2); A/sw/Hong Kong/5212/99 (H3N2); A/sw/Ille et Villaine/1455/99 (H1N1); A/sw/Italy/1654-1/99 (H1N2); A/sw/Italy/2034/99 (H1N1); A/sw/Italy/2064/99 (H1N2); A/sw/Berlin/1578/00 (H3N2); A/sw/Bakum/1832/00 (H1N2); A/sw/Bakum/1833/00 (H1N2); A/sw/Cote d'Armor/800/00 (H1N2); A/sw/Hong Kong/7982/00 (H3N2); A/sw/Italy/1081/00 (H1N2); A/sw/Belzig/2/01 (H1N1); A/sw/Belzig/54/01 (H3N2); A/sw/Hong Kong/9296/01 (H3N2); A/sw/Hong Kong/9745/01 (H3N2); A/sw/Spain/33601/01 (H3N2); A/sw/Hong Kong/1144/02 (H3N2); A/sw/Hong Kong/1197/02 (H3N2); A/sw/Spain/39139/02 (H3N2); A/sw/Spain/42386/02 (H3N2); A/Switzerland/8808/2002 (H1N1); A/sw/Bakum/1769/03 (H3N2); A/sw/Bissendorf/IDT 1864/03 (H3N2); A/sw/Ehren/IDT2570/03 (H1N2); A/sw/Gescher/IDT2702/03 (H1N2); A/sw/Haselünne/2617/03hp (H1N1); A/sw/Löningen/IDT2530/03 (H1N2); A/sw/IVD/IDT2674/03 (H1N2); A/sw/Nordkirchen/IDT1993/03 (H3N2); A/sw/Nordwalde/IDT2197/03 (H1N2); A/sw/Norden/IDT2308/03 (H1N2); A/sw/Spain/50047/03 (H1N1); A/sw/Spain/51915/03 (H1N1); A/sw/Vechta/2623/03 (H1N1); A/sw/Visbek/IDT2869/03 (H1N2); A/sw/Waltersdorf/IDT2527/03 (H1N2); A/sw/Damme/IDT2890/04 (H3N2); A/sw/Geldern/IDT2888/04 (H1N1); A/sw/Granstedt/IDT3475/04 (H1N2); A/sw/Greven/IDT2889/04 (H1N1); A/sw/Gudensberg/IDT2930/04 (H1N2); A/sw/Gudensberg/IDT2931/04 (H1N2); A/sw/Lohne/IDT3357/04 (H3N2); A/sw/Nortrup/IDT3685/04 (H1N2); A/sw/Seesen/IDT3055/04 (H3N2); A/sw/Spain/53207/04 (H1N1); A/sw/Spain/54008/04 (H3N2); A/sw/Stolzenau/IDT3296/04 (H1N2); A/sw/Wedel/IDT2965/04 (H1N1); A/sw/Bad Griesbach/IDT4191/05 (H3N2); A/sw/Cloppenburg/IDT4777/05 (H1N2); A/sw/Dötlingen/IDT3780/05 (H1N2); A/sw/Dötlingen/IDT4735/05 (H1N2); A/sw/Egglham/IDT5250/05 (H3N2); A/sw/Harkenblek/IDT4097/05 (H3N2); A/sw/Hertzen/IDT4317/05 (H3N2); A/sw/Krogel/IDT4192/05 (H1N1); A/sw/Laer/IDT3893/05 (H1N1); A/sw/Laer/IDT4126/05 (H3N2); A/sw/Merzen/IDT4114/05 (H3N2); A/sw/Muesleringen-S./IDT4263/05 (H3N2); A/sw/Osterhofen/IDT4004/05 (H3N2); A/sw/Sprenge/IDT3805/05 (H1N2); A/sw/Stadtlohn/IDT3853/05 (H1N2); A/sw/Voglarn/IDT4096/05 (H1N1); A/sw/Wohlerst/IDT4093/05 (H1N1); A/sw/Bad Griesbach/IDT5604/06 (H1N1); A/sw/Herzlake/IDT5335/06 (H3N2); A/sw/Herzlake/IDT5336/06 (H3N2); A/sw/Herzlake/IDT5337/06 (H3N2); and A/wild boar/Germany/R169/2006 (H3N2).

[0175] Other specific examples of strains of influenza A virus include, but are not limited to: A/Toronto/3141/2009 (H1N1); A/Regensburg/D6/2009 (H1N1); A/Bayern/62/2009 (H1N1); A/Bayern/62/2009 (H1N1); A/Bradenburg/19/2009 (H1N1); A/Bradenburg/20/2009 (H1N1); A/Distrito Federal/2611/2009 (H1N1); A/Mato Grosso/2329/2009 (H1N1); A/Sao Paulo/1454/2009 (H1N1); A/Sao Paulo/2233/2009 (H1N1); A/Stockholm/37/2009 (H1N1); A/Stockholm/41/2009 (H1N1); A/Stockholm/45/2009 (H1N1); A/swine/Alberta/OTH-33-1/2009 (H1N1); A/swine/Alberta/OTH-33-14/2009 (H1N1); A/swine/Alberta/OTH-33-2/2009 (H1N1); A/swine/Alberta/OTH-33-21/2009 (H1N1); A/swine/Alberta/OTH-33-22/2009 (H1N1); A/swine/Alberta/OTH-33-23/2009 (H1N1); A/swine/Alberta/OTH-33-24/2009 (H1N1); A/swine/Alberta/OTH-33-25/2009 (H1N1); A/swine/Alberta/OTH-33-3/2009 (H1N1); A/swine/Alberta/OTH-33-7/2009 (H1N1); A/Beijing/502/2009 (H1N1); A/Firenze/10/2009 (H1N1); A/Hong Kong/2369/2009 (H1N1); A/Italy/85/2009 (H1N1); A/Santo Domingo/572N/2009 (H1N1); A/Catalonial385/2009 (H1N1); A/Catalonia/386/2009 (H1N1); A/Catalonia/387/2009 (H1N1); A/Catalonia/390/2009 (H1N1); A/Catalonia/394/2009 (H1N1); A/Catalonia/397/2009 (H1N1); A/Catalonia/398/2009 (H1N1); A/Catalonia/399/2009 (H1N1); A/Sao Paulo/2303/2009 (H1N1); A/Akita/1/2009 (H1N1); A/Castro/JXP/2009 (H1N1); A/Fukushima/1/2009 (H1N1); A/Israel/276/2009 (H1N1); A/Israel/277/2009 (H1N1); A/Israel/70/2009 (H1N1); A/Iwate/1/2009 (H1N1); A/Iwate/2/2009 (H1N1); A/Kagoshima/1/2009 (H1N1); A/Osaka/180/2009 (H1N1); A/Puerto Montt/Bio87/2009 (H1N1); A/Sao Paulo/2303/2009 (H1N1); A/Sapporo/1/2009 (H1N1); A/Stockholm/30/2009 (H1N1); A/Stockholm/31/2009 (H1N1); A/Stockholm/32/2009 (H1N1); A/Stockholm/33/2009 (H1N1); A/Stockholm/34/2009 (H1N1); A/Stockholm/35/2009 (H1N1); A/Stockholm/36/2009 (H1N1); A/Stockholm/38/2009

(H1N1); A/Stockholm/39/2009 (H1N1); A/Stockholm/40/2009 (H1N1;) A/Stockholm/42/2009 (H1N1); A/Stockholm/43/2009 (H1N1); A/Stockholm/44/2009 (H1N1); A/Utsunomiya/2/2009 (H1N1); A/WRAIR/0573N/2009 (H1N1); and A/Zhejiang/DTID-ZJU01/2009 (H1N1).

[0176] In certain embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from an influenza B virus. In some embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1, a 5'CRS1 and a mORF from an influenza B virus. In specific embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from the same strain of influenza B virus. In certain embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a mORF, a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from the same strain of influenza B virus. In some embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1 and a 5' NCR1 from one influenza B virus strain and a 3' CRS1 and a 5'CRS1 from a different strain of influenza B virus. In some embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from one influenza B virus strain and a mORF from a strain of influenza B virus.

[0177] Non-limiting examples of influenza B viruses include strain Aichi/5/88, strain Akita/27/2001, strain Akita/5/2001, strain Alaska/16/2000, strain Alaska/1777/2005, strain Argentina/69/2001, strain Arizona/146/2005, strain Arizona/148/2005, strain Bangkok/163/90, strain Bangkok/34/99, strain Bangkok/460/03, strain Bangkok/54/99, strain Barcelona/215/03, strain Beijing/15/84, strain Beijing/184/93, strain Beijing/243/97, strain Beijing/43/75, strain Beijing/5/76, strain Beijing/76/98, strain Belgium/WV106/2002, strain Belgium/WV107/2002, strain Belgium/WV109/2002, strain Belgium/WV 114/2002, strain Belgium/WV122/2002, strain Bonn/43, strain Brazil/952/2001, strain Bucharest/795/03, strain Buenos Aires/161/00), strain Buenos Aires/9/95, strain Buenos Aires/SW16/97, strain Buenos Aires/VL518/99, strain Canada/464/2001, strain Canada/464/2002, strain Chaco/366/00, strain Chaco/R1 13/00, strain Cheju/303/03, strain Chiba/447/98, strain Chongqing/3/2000, strain clinical isolate SA1 Thailand/2002, strain clinical isolate SA10 Thailand/2002, strain clinical isolate SA100 Philippines/2002, strain clinical isolate SA101 Philippines/2002, strain clinical isolate SA110 Philippines/2002), strain clinical isolate SA112 Philippines/2002, strain clinical isolate SA113 Philippines/2002, strain clinical isolate SA114 Philippines/2002, strain clinical isolate SA2 Thailand/2002, strain clinical isolate SA20 Thailand/2002, strain clinical isolate SA38 Philippines/2002, strain clinical isolate SA39 Thailand/2002, strain clinical isolate SA99 Philippines/2002, strain CNIC/27/2001, strain Colorado/2597/2004, strain Cordoba/VA418/99, strain Czechoslovakia/16/89, strain Czechoslovakia/69/90, strain Daeku/10/97, strain Daeku/45/97, strain Daeku/47/97, strain Daeku/9/97, strain B/Du/4/78, strain B/Durban/39/98, strain Durban/43/98, strain Durban/44/98, strain B/Durban/52/98, strain Durban/55/98, strain Durban/56/98, strain England/1716/2005, strain England/2054/2005), strain England/23/04, strain Finland/154/2002, strain Finland/159/2002, strain Finland/160/2002, strain Finland/161/2002, strain Finland/162/03, strain Finland/162/2002, strain Finland/162/91, strain Finland/164/2003, strain Finland/172/91, strain Finland/173/2003, strain Finland/176/2003, strain Finland/184/91, strain Finland/188/2003, strain Finland/190/2003, strain Finland/220/2003, strain Finland/WV5/2002, strain Fujian/36/82, strain Geneva/5079/03, strain Genoa/11/02, strain Genoa/2/02, strain Genoa/21/02, strain Genova/54/02, strain Genoval55/02, strain Guangdong/05/94, strain Guangdong/08/93, strain Guangdong/5/94, strain Guangdong/55/89, strain Guangdong/8/93, strain Guangzhou/7/97, strain Guangzhou/86/92, strain Guangzhou/87/92, strain Gyeonggi/592/2005, strain Hannover/2/90, strain Harbin/07/94, strain Hawaii/10/2001, strain Hawaii/1990/2004, strain Hawaii/38/2001, strain Hawaii/9/2001, strain Hebei/19/94, strain Hebei/3/94), strain Henan/22/97, strain Hiroshima/23/2001, strain Hong Kong/110/99, strain Hong Kong/1115/2002, strain Hong Kong/112/2001, strain Hong Kong/123/2001, strain Hong Kong/1351/2002, strain Hong Kong/1434/2002, strain Hong Kong/147/99, strain Hong Kong/156/99, strain Hong Kong/157/99, strain Hong Kong/22/2001, strain Hong Kong/22/89, strain Hong Kong/336/2001, strain Hong Kong/666/2001, strain Hong Kong/9/89, strain Houston/1/91, strain Houston/1/96, strain Houston/2/96, strain Hunan/4/72, strain Ibaraki/2/85, strain ncheon/297/2005, strain India/3/89, strain Indial77276/2001, strain Israel/95/03, strain Israel/WV187/2002, strain Japan/1224/2005, strain Jiangsu/10/03, strain Johannesburg/1/99, strain Johannesburg/96/01, strain Kadoma/1076/99, strain Kadoma/122/99, strain Kagoshima/15/94, strain Kansas/22992/99, strain Khazkov/224/91, strain Kobe/1/2002, strain, strain Kouchi/193/99, strain Lazio/1/02, strain Lee/40, strain Leningrad/129/91, strain Lissabon/2/90), strain Los Angeles/1/02, strain Lusaka/270/99, strain Lyon/1271/96, strain Malaysia/83077/2001, strain Maputo/1/99, strain Mar del Plata/595/99, strain Maryland/1/01, strain Memphis/1/01, strain Memphis/12/97-MA, strain Michigan/22572/99, strain Mie/1/93, strain Milano/1/01, strain Minsk/318/90, strain Moscow/3/03, strain Nagoya/20/99, strain Nanchang/1/00, strain Nashville/107/93, strain Nashville/45/91, strain Nebraska/2/01, strain Netherland/801/90, strain Netherlands/429/98, strain New York/1/2002, strain NIB/48/90, strain Ningxia/45/83, strain Norway/1/84, strain Oman/16299/2001, strain Osaka/1059/97, strain Osaka/983/97-V2, strain Oslo/1329/2002, strain Oslo/1846/2002, strain Panama/45/90, strain Paris/329/90, strain Parma/23/02, strain Perth/211/2001, strain Peru/1364/2004, strain Philippines/5072/2001, strain Pusan/270/99, strain Quebec/173/98, strain Quebec/465/98, strain Quebec/7/01, strain Roma/1/03, strain Saga/S172/99, strain Seoul/13/95, strain Seoul/37/91, strain Shangdong/7/97, strain Shanghai/361/2002), strain Shiga/T30/98, strain Sichuan/379/99, strain

Singapore/222/79, strain Spain/WV27/2002, strain Stockholm/10/90, strain Switzerland/5441/90, strain Taiwan/0409/00, strain Taiwan/0722/02, strain Taiwan/97271/2001, strain Tehran/80/02, strain Tokyo/6/98, strain Trieste/28/02, strain Ulan Ude/4/02, strain United Kingdom/34304/99, strain USSR/100/83, strain Victoria/103/89, strain Vienna/1/99, strain Wuhan/356/2000, strain WV194/2002, strain Xuanwu/23/82, strain Yamagata/1311/2003, strain Yamagata/K500/2001, strain Alaska/12/96, strain GA/86, strain NAGASAKI/1/87, strain Tokyo/942/96, and strain Rochester/02/2001.

[0178] In certain embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from an influenza C virus. In some embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1, a 5'CRS1 and a mORF from an influenza C virus. In specific embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from the same strain of influenza C virus. In certain embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a mORF, a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from the same strain of influenza C virus. In some embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1 and a 5' NCR1 from one influenza C virus strain and a 3' CRS1 and a 5'CRS1 from a different strain of influenza C virus. In some embodiments, a recombinant influenza virus comprises a chimeric influenza virus gene segment that includes a 3' NCR1, a 5' NCR1, a 3' CRS1 and a 5'CRS1 from one influenza C virus strain and a mORF from a strain of influenza C virus.

[0179] Non-limiting examples of influenza C viruses include strain Aichi/1/81, strain Ann Arbor/1/50, strain Aomori/74, strain California/78, strain England/83, strain Greece/79, strain Hiroshima/246/2000, strain Hiroshimal252/2000, strain Hyogo/1/83, strain Johannesburg/66, strain Kanagawa/1/76, strain Kyoto/1/79, strain Mississippi/80, strain Miyagi/1/97, strain Miyagi/5/2000, strain Miyagi/9/96, strain Nara/2/85, strain NewJersey/76, strain pig/Beijing/115/81, strain Saitama/3/2000), strain Shizuoka/79, strain Yamagata/2/98, strain Yamagata/6/2000, strain Yamagata/9/96, strain BERLIN/1/85, strain ENGLAND/892/8, strain GREAT LAKES/1167/54, strain JJ/50, strain PIG/BEIJING/10/81, strain PIG/BEIJING/439/82), strain TAYLOR/1233/47, and strain C/YAMAGATA/10/81.

[0180] In certain embodiments, when a recombinant influenza virus described herein comprises a group of cosegregating chimeric influenza virus gene segments that includes less than the full set of gene segments found in a genome of an influenza virus (*i.e.*, less than the eight types of gene segments for an influenza A virus, less than eight types of gene segments for an influenza B virus, or less than the seven types of gene segments for an influenza C virus), the virus further comprises gene segments to complete the full set of gene segments found in a genome of an influenza virus. For example, if a recombinant influenza virus comprises a chimeric influenza virus gene segment that encodes an HA protein and a chimeric influenza virus gene segment that encodes a PA protein, the recombinant influenza may further comprise NS, PB1, PB2, M, NP, and NA (for influenza A and B viruses) influenza virus gene segments or derivatives thereof. The influenza virus gene segments or derivatives thereof that complete the full set of gene segments found in a genome of an influenza virus are referred to herein as "complementing influenza virus gene segments." By way of example and not by limitation, a recombinant influenza virus may comprise the following gene segments:

Table 16

Chimeric Influenza Virus Gene Segment Derived From:	Complementing Influenza Virus Gene Segments
HA, NS	PB2, PB1, PA, NP, NA, M
HA, NA	PB2, PB1, PA, NP, NS, M
NA, NS	PB2, PB1, PA, HA, NP, M
HA, NA, NS	PB2, PB1, PA, NP, M
HA, PB1, PB2	PA, NP, NS, M, NA
HA, PB1, PB2, NS	PA, NP, M, NA
HA, PB1, PB2, PA	NS, NP, M, NA
HA, PA, NS	PB1, PB2, NP, M, NA
HA, M, NS	PB1, PB2, PA, M, NA
HA, PA, PB1, PB2, PA	M, NA, NS
NS, PB1, PB2, PA	HA, M, NA, NP
HA, NA, PA, NS	NP, PB1, PB2,
HA, NA, NS	NP, PA, PB1, PB2

(continued)

Chimeric Influenza Virus Gene Segment Derived From:	Complementing Influenza Virus Gene Segments
HA, NP, PB1, PB2	M, NA, NS, PA

[0181] In certain embodiments, the complementing influenza virus gene segments may all be derived from the same type or subtype of an influenza virus. In other embodiments, the complementing influenza virus gene segments may be derived from one, two or more different types or subtypes of an influenza virus. In some embodiments, the complementing influenza virus gene segments may all be derived from the same strain of an influenza virus. In other embodiments, the complementing influenza virus gene segments may be derived from one, two or more different strains of an influenza virus. In certain embodiments, the complementing influenza virus gene segments can be derived from an attenuated influenza virus strain.

[0182] In certain embodiments, one, two or more chimeric influenza virus gene segments and one, two or more of the complementing influenza virus gene segments may be derived from the same type or subtype of an influenza virus. In other embodiments, one, two or more chimeric influenza virus gene segments and one, two or more of the complementing influenza virus gene segments may be derived from one, two or more different types or subtypes of an influenza virus. In some embodiments, one, two or more chimeric influenza virus gene segments and one, two or more of the complementing influenza virus gene segments may be derived from the same strain of an influenza virus. In other embodiments, one, two or more chimeric influenza virus gene segments and one, two or more of the complementing influenza virus gene segments may be derived from one, two or more different strains of an influenza virus.

[0183] In certain embodiments, a recombinant influenza virus described herein comprises at least one gene segment that encodes a fusion protein. The fusion protein can be encoded by a chimeric influenza virus gene segment or a complementing influenza virus gene segment. A fusion protein can be a fusion of an influenza virus protein or a fragment thereof with a heterologous protein (such as a viral antigen, a bacterial antigen, a parasitic antigen, a fungal antigen, a tumor antigen, a tumor associated antigen, a cytokine, a growth factor, a peptide tag, or a detectable substance (see Section 5.1.3 for examples of such antigens, cytokines, growth factors, peptide tags, and detectable substances))

[0184] In certain embodiments, a recombinant influenza virus comprises nine gene segments, wherein (a) at least one gene segment comprises: (i) the packaging signals found in the 3' non-coding region of a first type of influenza virus gene segment or a derivative thereof; (ii) the packaging signals found in the 3' proximal coding region of the first type of influenza virus gene segment or a derivative thereof, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) an open reading frame of a second type of influenza virus gene segment or a fragment or a derivative thereof, wherein the 3' and 5' proximal nucleotides in the open reading frame are mutated; (iv) the packaging signals found in the 5' proximal coding region of the first type of influenza virus gene segment or a derivative thereof; and (v) the packaging signals found in the 5' non-coding region of the first type of influenza virus gene segment or a derivative thereof; and (b) at least one gene segment comprises: (i) the packaging signals found in the 3' non-coding region of the second type of influenza virus gene segment or a derivative thereof; (ii) the packaging signals found in the 3' proximal coding region of the second type of influenza virus gene segment or a derivative thereof, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) an open reading frame heterologous to 1, 2, 3, 4, 5, 6, 7 or 8 of the influenza virus gene segments; (iv) the packaging signals found in the 5' proximal coding region of the second type of influenza virus gene segment or a derivative thereof; and (v) the packaging signals found in the 5' non-coding region of the second type of influenza virus gene segment or a derivative thereof. In other embodiments, the 3' and/or the 5' proximal coding region sequences flank the open reading frame and are not translated. In some embodiments, the 3' proximal coding region sequence has been mutated so as to preclude the translation of the 3' proximal coding region sequence. In some embodiments, the 5 ' proximal coding region sequence has one or more mutations so as to ensure that the 5' proximal coding region sequence is not translated. In a specific embodiment, the mutations introduced into the open reading frame of the influenza virus gene segment or a fragment are silent mutations. See, e.g., Examples 2 and 3 and Figures 29 and 30 for examples of nine-segmented recombinant influenza viruses. In certain embodiments, the nine-segmented recombinant influenza virus is attenuated.

[0185] In another embodiment, a recombinant influenza virus comprises nine gene segments, wherein: (a) at least one of the gene segments comprises: (i) the 3' non-coding region of a first type of influenza virus gene segment; (ii) a 3' proximal coding region of the first type of influenza virus gene segment, wherein any start codon in the 3' proximal coding region of the first type of influenza virus gene segment is mutated; (iii) an open reading frame of a second type of influenza virus gene segment, wherein a certain number of the 3' proximal nucleotides and a certain number of the 5' proximal nucleotides have been mutated; and (v) a 5' proximal coding region of the first type of influenza virus gene segment; and (vi) the 5' non-coding region of the first type of influenza virus gene segment; and (b) at least one gene segment comprises: (i) the 3' non-coding region of the second type of influenza virus gene segment; (ii) a 3' proximal coding region of the second type of influenza virus gene segment, wherein any start codon in the 3' proximal coding

region of the second type of influenza virus gene segment is mutated; (iii) an open reading frame heterologous to 1, 2, 3, 4, 5, 6, 7 or 8 of the influenza virus gene segments; and (v) a 5' proximal coding region of the second type of influenza virus gene segment; and (vi) the 5' non-coding region of the second type of influenza virus gene segment. In certain embodiments, 5 to 25 or 5 to 50 of the 3' proximal nucleotides and 5 to 25 or 5 to 50 of the 5' proximal nucleotides of the open reading frame of the second influenza virus gene segment carry one or more mutations. In a specific embodiment, such mutations are silent mutations. In some embodiments, the 5' proximal coding regions of the first and second influenza virus gene segment are mutated so that the 5' proximal coding regions are not translated.

[0186] In some embodiments, the nine-segmented recombinant influenza virus encodes and/or expresses influenza virus antigens from two different types, subtypes or strains of influenza virus. In a specific embodiment, the recombinant influenza virus encodes and/or expresses HA antigens from two different types, subtypes or strains of influenza virus. For example, the nine-segmented recombinant influenza virus encodes and/or expresses an H1 HA and an H3 HA antigen. In some embodiments, the one HA antigens is from a seasonal influenza virus and the other HA antigen is from a pandemic influenza virus. In specific embodiments, each of the two HA antigens may comprise an attenuating mutation. In certain embodiments, the nine-segmented recombinant influenza virus encodes and/or expresses influenza virus antigens and at least one, two, three or four, or 1 to 3, 1 to 4, or 2 to 4 non-influenza virus antigens (*e.g.*, antigens from bacterial pathogens, or viral pathogens other an influenza virus). In accordance with these embodiments, in some embodiments, the heterologous open reading frame of the one gene segment can encode an influenza virus antigen from a different type, subtype or strain of influenza virus than the influenza virus antigens encoded by the other gene segments. In other embodiments, the heterologous open reading frame of the one gene segment can encode a non-influenza virus antigen (*e.g.*, a bacterial antigen, tumor antigen, or viral antigen other than an influenza virus antigen). In yet other embodiments, the heterologous open reading frame encodes a detectable protein, such as, e.g., GFP or luciferase.

[0187] In certain embodiments, a recombinant influenza virus described herein comprises at least one gene segment that encodes a bicistronic mRNA. The biscistronic mRNA can be encoded by a chimeric influenza virus gene segment or a complementing influenza virus gene segment. Techniques for creating an influenza virus gene segment that encodes a bicistronic mRNA are known in the art. Bicistronic techniques allow the engineering of coding sequences of multiple proteins into a single mRNA through the use of internal ribosome entry site (IRES) sequences. Briefly, a coding region of one protein is inserted into the open reading frame of a second protein. The insertion is flanked by an IRES and any untranslated signal sequences necessary for proper expression and/or function. The insertion must not disrupt the open reading frame, polyadenylation or transcriptional promoters of the second protein (see, *e.g.* , Garcia-Sastre et al., 1994, J. Virol. 68:6254-6261 and Garcia-Sastre et al., 1994 Dev. Biol. Stand. 82:237-246 See also, *e.g.*, U.S. Patent No. 6,887,699, U.S. Patent No. 6,001,634, U.S. Patent No. 5,854,037 and U.S. Patent No. 5,820,871. Any IRES known in the art or described herein may be used in accordance with the invention (*e.g.*, the IRES of BiP gene, nucleotides 372 to 592 of GenBank database entry HUMGRP78; or the IRES of encephalomyocarditis virus (EMCV), nucleotides 1430-2115 of GenBank database entry CQ867238.). One of the open reading frames of the bicistronic mRNA may encode an influenza virus protein or a fragment thereof and the other open reading frame of the bicistronic mRNA may encode a heterologous protein (such as a viral antigen, a bacterial antigen, a parasitic antigen, a fungal antigen, a tumor antigen, a tumor associated antigen, a cytokine, a growth factor, a peptide tag, or a detectable substance (see Section 5.1.3 for examples of such antigens, cytokines, growth factors, peptide tags, and detectable substances)).

[0188] In specific embodiments, a recombinant influenza virus described herein is attenuated. In a particular embodiment, the recombinant influenza virus is attenuated such that the virus remains, at least partially, infectious and can replicate *in vivo,* but only generate low titers resulting in subclinical levels of infection that are non-pathogenic. Such attenuated viruses are especially suited for embodiments described herein wherein the virus or an immunogenic composition thereof is administered to a subject to induce an immune response.

[0189] In some embodiments, a recombinant influenza virus described herein comprises one or more attenuating mutations in a chimeric influenza virus gene segment. In certain embodiments, a recombinant influenza virus described herein comprises one or more attenuating mutations in two, three or more chimeric influenza virus gene segments. In some embodiments, a recombinant influenza virus described herein comprises one or more attenuating mutations in a complementing influenza virus gene segment. In certain embodiments, a recombinant influenza virus described herein comprises one or more attenuating mutations in two, three or more complementing influenza virus gene segments. In some embodiments, a recombinant influenza virus described herein comprises one or more attenuating mutations in a chimeric influenza virus gene segment and one or more attenuating mutations in a complementing influenza virus gene segment. In certain embodiments, a recombinant influenza virus described herein comprises one or more attenuating mutations in one, two, three or more chimeric influenza virus gene segments and one or more attenuating mutations in one, two, three or more complementing influenza virus gene segments.

[0190] In certain embodiments, the one or more attenuating mutations may be in the open reading frame of a gene segment encoding one or more of the following: NS 1, NP, HA, NA, PB1, PB2 and/or PA. In a specific embodiment, the one or more attenuating mutations may be in the open reading frame of an HA gene segment. In another specific

embodiment, the one or more attenuating mutations may be in the open reading of an NP gene segment. In another embodiment, the one or more attenuating mutations may be in the open reading frame of an PB1 gene segment In another embodiment, the one or more attenuating mutations may be in the open reading frame of an PB2 gene segment. In certain embodiments, the one or more attenuating mutations in a gene segment of an influenza virus can be accomplished according to any method known in the art, such as, *e.g.*, selecting viral mutants generated by chemical mutagenesis, mutation of the genome by genetic engineering, selecting reassortant viruses that contain segments with attenuated function, or selecting for conditional virus mutants (*e.g.*, cold-adapted viruses such as A/Leningrad/134/47/57 (H2N2), A/Ann Arbor/6/60 (H2N2), B/Ann Arbor/1/66, and B/Lee/40). In a specific embodiment, one or more temperature sensitive mutations that are attenuating may be introduced in an open reading frame of a gene segment. In some embodiments, the one or more temperature sensitive mutations include one or more of the following: PB1 (K391E, E581G, A661T), PB2 (N265S), and NP (D34G).

[0191] In some embodiments, an attenuated recombinant influenza virus expresses the following NP, PB1 and PB2 proteins encoded by cold adapted vaccine master strain A/Ann Arbor/6/60 (see, *e.g.*, Jin et al., 2003, Virology 306: 18-24 for a description of the virus).

[0192] In some embodiments, an attenuated recombinant influenza virus expresses a mutated NS 1 protein that impairs the ability of the virus to antagonize the cellular interferon (IFN) response. Examples of the types of mutations that can be introduced into the open reading frame of influenza virus NS1 include deletions, substitutions, insertions and combinations thereof. One or more mutations can be introduced anywhere throughout the open reading frame of NS1 (*e.g.*, the N-terminus, the C-terminus or somewhere in between) and/or the regulatory elements of the NS1 gene. In one embodiment, an attenuated recombinant influenza virus comprises a genome having a mutation in an influenza virus NS1 open reading frame resulting in a deletion consisting of 5, preferably 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 75, 80, 85, 90, 95, 99, 100, 105, 110, 115, 120, 125, 126, 130, 135, 140, 145, 150, 155, 160, 165, 170 or 175 amino acid residues from the C-terminus of NS1, or a deletion of between 5-170, 25-170, 50-170, 100-170, 100-160, or 105-160 amino acid residues from the C-terminus. In another embodiment, a recombinant attenuated influenza virus comprises a genome having a mutation in an influenza virus NS1 open reading frame such that it encodes an NS1 protein of amino acid residues 1-130, amino acid residues 1-126, amino acid residues 1-125, amino acid residues 1-124, amino acid residues 1-120, amino acid residues 1-115, amino acid residues 1-110, amino acid residues 1-100, amino acid residues 1-99, amino acid residues 1-95, amino acid residues 1-85, amino acid residues 1-83, amino acid residues 1-80, amino acid residues 1-75, amino acid residues 1-73, amino acid residues 1-70, amino acid residues 1-65, or amino acid residues 1-60, wherein the N-terminus amino acid is number 1. For examples of NS1 mutations and influenza viruses comprising a mutated NS1, see, *e.g.*, U.S. Patent Nos. 6,468,544 and 6,669,943; and Li et al., 1999, J. Infect. Dis. 179:1132-1138.

[0193] In some embodiments, an attenuated recombinant influenza virus expresses a mutated M2 protein such as described by Watanabe et al., 2008, J. Virol. 82(5): 2486-2492.

[0194] In a specific embodiment, an attenuated recombinant influenza virus comprises a first chimeric influenza virus gene segment encoding an HA from a pandemic or seasonal influenza virus and a second chimeric influenza virus gene segment encoding a viral polymerase subunit (*i.e.*, *e.g.*, PA, PB1 or PB2) with one or more attenuating mutations.

5.3 CONSTRUCTION OF INFLUENZA VIRUS

[0195] Techniques known to one skilled in the art may be used to produce a recombinant influenza virus of the invention containing two or more chimeric influenza virus gene segments described herein. For example, reverse genetics techniques may be used to generate such an influenza virus. Briefly, reverse genetics techniques generally involve the preparation of synthetic recombinant viral RNAs that contain the non-coding regions of the negative-strand, viral RNA which are essential for the recognition by viral polymerases and for packaging signals necessary to generate a mature virion. The recombinant RNAs are synthesized from a recombinant DNA template and reconstituted in vitro with purified viral polymerase complex to form recombinant ribonucleoproteins (RNPs) which can be used to transfect cells. A more efficient transfection is achieved if the viral polymerase proteins are present during transcription of the synthetic RNAs either *in vitro* or *in vivo.* The synthetic recombinant RNPs can be rescued into infectious virus particles. The foregoing techniques are described in U.S. Patent No. 5,166,057 issued November 24, 1992; in U.S. Patent No. 5,854,037 issued December 29, 1998; in European Patent Publication EP 0702085A1, published February 20, 1996; in U.S. Patent Application Serial No. 09/152,845; in International Patent Publications PCT WO 97/12032 published April 3, 1997; WO 96/34625 published November 7, 1996; in European Patent Publication EP A780475; WO 99/02657 published January 21, 1999; WO 98/53078 published November 26, 1998; WO 98/02530 published January 22, 1998; WO 99/15672 published April 1, 1999; WO 98/13501 published April 2, 1998; WO 97/06270 published February 20, 1997; and EPO 780 475A1 published June 25, 1997.

[0196] Alternatively, helper-free plasmid technology may be used to produce a recombinant influenza virus containing one or more chimeric influenza virus gene segments. Briefly, full length cDNAs of viral segments are amplified using

PCR with primers that include unique restriction sites, which allow the insertion of the PCR product into the plasmid vector (Flandorfer et al., 2003, J. Virol. 77:9116-9123; Nakaya et al., 2001, J. Virol. 75:11868-11873. The plasmid vector is designed so that an exact negative (vRNA sense) transcript is expressed. For example, the plasmid vector may be designed to position the PCR product between a truncated human RNA polymerase I promoter and a hepatitis delta virus ribozyme sequence such that an exact negative (vRNA sense) transcript is produced from the polymerase I promoter. Separate plasmid vectors comprising each viral segment as well as expression vectors comprising necessary viral proteins may be transfected into cells leading to production of recombinant viral particles. In another example, plasmid vectors from which both the viral genomic RNA and mRNA encoding the necessary viral proteins are expressed may be used. For a detailed description of helper-free plasmid technology see, *e.g.*, International Publication No. WO 01/04333; U.S. Patent Nos. 6,951,754, 7,384,774, 6,649,372, and 7,312,064; Fodor et al., 1999, J. Virol. 73:9679-9682; Quinlivan et al., 2005, J. Virol. 79:8431-8439; Hoffmann et al., 2000, Proc. Natl. Acad. Sci. USA 97:6108-6113; and Neumann et al., 1999, Proc. Natl. Acad. Sci. USA 96:9345-935.

[0197] In specific embodiments, one, two or more nucleic acid sequences encoding one, two or more chimeric influenza virus gene segments or the complements thereof are transfected into a host cell that provides the remainder of the gene segments found in an influenza virus genome and expresses the proteins necessary for production of viral particles. Techniques known in the art can be used to isolate/purify the recombinant influenza virus that results (see, *e.g.*, Section 5.4, *infra* for techniques for isolation/purification of influenza virus).

5.4 PROPAGATION OF INFLUENZA VIRUS

[0198] The recombinant influenza viruses provided herein can be propagated in any substrate that allows the virus to grow to titers that permit the uses of the viruses provided herein. In one embodiment, the substrate allows the recombinant influenza viruses described herein to grow to titers comparable to those determined for the corresponding wild-type viruses.

[0199] The recombinant influenza virus provided herein may be grown in host cells (*e.g.*, avian cells, chicken cells, etc.) that are susceptible to infection by the viruses, embryonated eggs or animals (*e.g.*, birds). Specific examples of host cells include Vero cells, MDCK cells, MBCK cells, COS cells, 293 cells, 293T cells, A549 cells, MDBK cells, etc. Such methods are well-known to those skilled in the art. In a specific embodiment, the recombinant influenza viruses described herein may be propagated in cell lines. In another embodiment, the recombinant influenza viruses described herein described herein are propagated in chicken cells or embryonated eggs. Representative chicken cells include, but are not limited to, chicken embryo fibroblasts and chicken embryo kidney cells.

[0200] The recombinant influenza viruses provided herein may be propagated in embryonated eggs, *e.g.*, from 6 to 14 days old, 6 to 9 days old, 10 to 12 days old, or 10 to 14 days old. Young or immature embryonated eggs can be used to propagate the recombinant influenza viruses Immature embryonated eggs encompass eggs which are less than ten day old eggs, *e.g.*, eggs 6 to 9 days that are interferon (IFN)-deficient. Immature embryonated eggs also encompass eggs which artificially mimic immature eggs up to, but less than ten day old, as a result of alterations to the growth conditions, *e.g.*, changes in incubation temperatures; treating with drugs; or any other alteration which results in an egg with a retarded development, such that the IFN system is not fully developed as compared with ten to twelve day old eggs. In one embodiment, the recombinant influenza viruses may be propagated in 10 day old embryonated eggs. The recombinant influenza viruses can be propagated in different locations of the embryonated egg, *e.g.*, the allantoic cavity. In a specific embodiment, the embryonated egg is an embryonated chicken egg. For a detailed discussion on the growth and propagation viruses, see, *e.g.*, U.S. Patent No. 6,852,522 and U.S. Patent No. 6,852,522.

[0201] For virus isolation, the recombinant influenza viruses provided herein can be removed from cell culture and separated from cellular components, typically by well known clarification procedures, *e.g.*, such as gradient centrifugation and column chromatography, and may be further purified as desired using procedures well known to those skilled in the art, *e.g.*, plaque assays.

5.5 COMPOSITIONS & ROUTES OF ADMINISTRATION

[0202] The recombinant influenza viruses provided herein may be incorporated into compositions. In a specific embodiment, the compositions are pharmaceutical compositions, such as immunogenic compositions (*e.g.*, vaccine formulations). The pharmaceutical compositions provided herein can be in any form that allows for the composition to be administered to a subject. In a specific embodiment, the pharmaceutical compositions are suitable for veterinary and/or human administration. The compositions may be used in methods of preventing and/or treating an influenza virus infection. The compositions may also be used in methods or preventing and/or treating influenza virus disease.

[0203] In one embodiment, a pharmaceutical composition comprises a recombinant influenza virus in an admixture with a pharmaceutically acceptable carrier. In some embodiments, a pharmaceutical composition may comprise one or more other therapies in addition to a recombinant influenza virus. In specific embodiments, a recombinant influenza

virus provided herein that is incorporated into a pharmaceutical composition (*e.g.*, an immunogenic composition such as a vaccine) is a live virus. An immunogenic composition comprising a live recombinant influenza virus for administration to a subject may be preferred because multiplication of the virus in the subject may lead to a prolonged stimulus of similar kind and magnitude to that occurring in natural infections, and therefore, confer substantial, long lasting immunity.

[0204] In some embodiments, a recombinant influenza virus provided herein that is incorporated into a pharmaceutical composition (*e.g.*, an immunogenic composition such as a vaccine) is inactivated. Techniques known to one of skill in the art may be used to inactivate recombinant influenza viruses described herein. Common methods use formalin, heat, or detergent for inactivation. See, *e.g.*, U.S. Patent No. 6,635,246, Other methods include those described in U.S. Patent Nos. 5,891,705; 5,106,619 and 4,693,981.

[0205] In specific embodiments, immunogenic compositions are monovalent formulations. In other embodiments, immunogenic compositions are multivalent formulations. In one example, a multivalent formulation comprises one or more recombinant influenza viruses that expresses antigens from an influenza A virus and one or more recombinant influenza viruses that expresses antigens from an influenza B virus.

[0206] In a specific embodiment, an immunogenic composition comprises a recombinant influenza virus provided herein which contains nine gene segments. In certain embodiments, such a nine-segmented influenza virus expresses influenza virus antigens from two different types, subtypes, or strains of influenza virus. In a specific embodiment, the nine-segmented recombinant influenza virus expresses HA antigens from two different types, subtypes, or strains of influenza virus. In some embodiments, the nine-segmented influenza virus expresses influenza virus antigens and at least one, two, three, or four or 1 to 3, 1 to 4, or 2 to 4 non-influenza virus antigens.

[0207] As used herein, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeiae for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the pharmaceutical composition is administered. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

[0208] In certain embodiments, biodegradable polymers, such as ethylene vinyl acetate, polyanhydrides, polyethylene glycol (PEGylation), polymethyl methacrylate polymers, polylactides, poly(lactide-co-glycolides), polyglycolic acid, collagen, polyorthoesters, and polylactic acid, may be used as carriers. Liposomes or micelles can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

[0209] In a specific embodiment, pharmaceutical compositions are formulated to be suitable for the intended route of administration to a subject. For example, the pharmaceutical composition may be formulated to be suitable for parenteral, oral, intradermal, intransal, transdermal, pulmonary, colorectal, intraperitoneal, and rectal administration. In a specific embodiment, the pharmaceutical composition may be formulated for intravenous, oral, intraperitoneal, intranasal, intratracheal, subcutaneous, intramuscular, topical, intradermal, transdermal or pulmonary administration.

[0210] In certain embodiments, the compositions provided herein comprise, or are administered in combination with, an adjuvant. The adjuvant for administration in combination with a composition provided herein may be administered before, concommitantly with, or after administration of the composition. In specific embodiments, an inactivated virus immunogenic composition provided herein comprises one or more adjuvants. In some embodiments, the term "adjuvant" refers to a compound that when administered in conjunction with or as part of a composition described herein augments, enhances and/or boosts the immune response to a recombinant influenza virus, but when the compound is administered alone does not generate an immune response to the virus. In some embodiments, the adjuvant generates an immune response to a recombinant influenza virus and does not produce an allergy or other adverse reaction. Adjuvants can enhance an immune response by several mechanisms including, *e.g.*, lymphocyte recruitment, stimulation of B and/or T cells, and stimulation of macrophages.

[0211] Specific examples of adjuvants include, but are not limited to, aluminum salts (alum) (such as aluminum hydroxide, aluminum phosphate, and aluminum sulfate), 3 De-O-acylated monophosphoryl lipid A (MPL) (see GB 2220211), MF59 (Novartis), AS03 (GlaxoSmithKline), AS04 (GlaxoSmithKline), polysorbate 80 (Tween 80; ICL Americas, Inc.), imidazopyridine compounds (see International Application No. PCT/US2007/064857, published as International Publication No. WO2007/109812), imidazoquinoxaline compounds (see International Application No. PCT/US2007/064858, published as International Publication No. WO2007/109813) and saponins, such as QS21 (see Kensil et al., in Vaccine Design: The Subunit and Adjuvant Approach (eds. Powell & Newman, Plenum Press, NY, 1995); U.S. Pat. No. 5,057,540). In some embodiments, the adjuvant is Freund's adjuvant (complete or incomplete). Other adjuvants are oil in water emulsions (such as squalene or peanut oil), optionally in combination with immune stimulants, such as monophosphoryl lipid A (see Stoute et al., N. Engl. J. Med. 336, 86-91 (1997)). Another adjuvant is CpG (Bioworld Today, Nov. 15, 1998). Such adjuvants can be used with or without other specific immunostimulating agents such as MPL or 3-DMP, QS21,

polymeric or monomeric amino acids such as polyglutamic acid or polylysine..

[0212] The pharmaceutical compositions provided herein can be included in a container, pack, or dispenser together with instructions for administration.

5.5.1. LIVE VIRUS VACCINES

[0213] In one embodiment, provided herein are immunogenic compositions (*e.g.*, vaccines) comprising one or more live recombinant influenza viruses provided herein. In some embodiments, the live virus is attenuated. In some embodiments, an immunogenic composition comprises two, three, four or more live viruses.

[0214] In certain embodiments, provided herein are immunogenic compositions (*e.g.*, vaccines) comprising about 10^5 to about 10^{10} fluorescent focus units (FFU) of live attenuated recombinant influenza virus described herein, about 0.1 to about 0.5 mg monosodium glutamate, about 1.0 to about 5.0 mg hydrolyzed procine gelatin, about 1.0 to about 5.0 mg arginine, about 10 to about 15 mg sucrose, about 1.0 to about 5.0 mg dibasic potassium phosphate, about 0.5 to about 2.0 mg monobasic potassium phosphate, and about 0.001 to about 0.05 μg/ml gentamicin sulfate per dose. In some embodiments, the immunogenic compositions (*e.g.*, vaccines) are packaged as pre-filled sprayers containing single 0.2 ml doses.

[0215] In a specific embodiment, provided herein are immunogenic compositions (*e.g.*, vaccines) comprising $10^{6.5}$ to $10^{7.5}$ FFU of live attenuated recombinant influenza virus described herein, 0.188 mg monosodium glutamate, 2.0 mg hydrolyzed procine gelatin, 2.42 mg arginine, 13.68 mg sucrose, 2.26 mg dibasic potassium phosphate, 0.96 mg monobasic potassium phosphate, and < 0.015 μg/ml gentamicin sulfate per dose. In some embodiments, the immunogenic compositions (*e.g.*, vaccines) are packaged as pre-filled sprayers containing single 0.2 ml doses.

[0216] In a specific embodiment, the live virus is propagated in embryonated chicken eggs before its use in an immunogenic composition described herein. In another specific embodiment, the live virus is not propagated in embryonated chicken eggs before its use in an immunogenic composition described herein. In another specific embodiment, the live virus is propagated in mammalian cells, e.g., immortalized human cells (see, e.g., International Application No. PCT/EP2006/067566 published as International Publication No. WO 07/045674 or canine kidney cells such as MDCK cells (see, e.g., International Application No. PCT/IB2007/003536 published as International Publication No. WO 08/032219 before its use in an immunogenic composition described herein.

[0217] An immunogenic composition comprising a live virus for administration to a subject may be preferred because multiplication of the virus in the subject may lead to a prolonged stimulus of similar kind and magnitude to that occurring in natural infections, and therefore, confer substantial, long lasting immunity.

5.6 GENERATION OF ANTIBODIES THAT SPECIFICALLY BIND TO INFLUENZA VIRUS

[0218] The recombinant influenza viruses described herein may be used to elicit neutralizing antibodies against influenza, for example, against influenza virus hemagglutinin. In a specific case, a recombinant influenza virus described herein or a composition thereof may be administered to a non-human subject (*e.g.*, a mouse, rabbit, rat, guinea pig, etc.) to induce an immune response that includes the production of antibodies which may be isolated using techniques known to one of skill in the art (*e.g.*, immunoaffinity chromatography, centrifugation, precipitation, etc.).

[0219] In certain cases, the non-human subjects administered a recombinant influenza virus described herein or an immunogenic composition thereof in accordance with the methods described herein are transgenic animals (e.g., transgenic mice) that are capable of producing human antibodies. Human antibodies can be produced using transgenic mice which are incapable of expressing functional endogenous immunoglobulins, but which can express human immunoglobulin genes. For example, the human heavy and light chain immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. Alternatively, the human variable region, constant region, and diversity region may be introduced into mouse embryonic stem cells in addition to the human heavy and light chain genes. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, see Lonberg and Huszar, Int. Rev. Immunol. 13:65-93 (1995). For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, see, *e.g.*, PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Pat. Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; and 5,939,598. Companies such as Abgenix, Inc. (Freemont, Calif.), Gen-

pharm (San Jose, Calif.), and Medarex, Inc. (Princeton, N.J.) can be engaged to provide human antibodies directed against a selected antigen.

[0220] Alternatively, a recombinant influenza virus described herein may be used to screen for antibodies from antibody libraries. For example, a recombinant influenza virus may be immobilized to a solid support (*e.g.*, a silica gel, a resin, a derivatized plastic film, a glass bead, cotton, a plastic bead, a polystyrene bead, an alumina gel, or a polysaccharide, a magnetic bead), and screened for binding to antibodies. As an alternative, the antibodies may be immobilized to a solid support and screened for binding to a recombinant influenza virus described herein. Any screening assay, such as a panning assay, ELISA, surface plasmon resonance, or other antibody screening assay known in the art may be used to screen for antibodies that bind to a recombinant influenza virus. The antibody library screened may be a commercially available antibody library, an *in vitro* generated library, or a library obtained by identifying and cloning or isolating antibodies from an individual infected with influenza. In particular embodiments, the antibody library is generated from a survivor of an influenza virus outbreak. Antibody libraries may be generated in accordance with methods known in the art. In a particular embodiment, the antibody library is generated by cloning the antibodies and using them in phage display libraries or a phagemid display library.

[0221] Antibodies elicited or identified in accordance with the methods described herein may be tested for specificity for influenza virus antigens and the ability to neutralize influenza virus using the biological assays known in the art or described herein. In one case, an antibody identified or isolated from a non-human animal antibody specifically binds to an influenza virus antigen. In another case, an antibody identified or isolated from a non-human animal specifically binds to an influenza virus antigen expressed by two or more types, subtypes or strains of influenza virus. In one case, an antibody identified or isolated from a non-human animal neutralizes one, two or more influenza virus types, subtypes or strains. In some cases, an antibody elicited or identified using a recombinant influenza virus described herein neutralizes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 or more subtypes or strains of influenza virus. In one embodiment, the neutralizing antibody neutralizes one or more strains or subtypes of influenza A viruses. In another case, the neutralizing antibody neutralizes one or more strains of influenza B viruses. In another embodiment, the neutralizing antibody neutralizes one or more strains of influenza A virus and one or more strains of influenza B viruses.

[0222] Antibodies elicited or identified using a recombinant influenza virus described herein include immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that specifically binds to a hemagglutinin polypeptide. The immunoglobulin molecules may be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule. Antibodies include, but are not limited to, monoclonal antibodies, multispecific antibodies, human antibodies, humanized antibodies, chimeric antibodies, single-chain Fvs (scFv), single chain antibodies, Fab fragments, F(ab') fragments, disulfide-linked Fvs (sdFv), and anti-idiotypic (anti-Id) antibodies (including, *e.g.*, anti-Id antibodies to antibodies elicited or identified using a method described herein), and epitope-binding fragments of any of the above.

[0223] Antibodies elicited or identified using a recombinant influenza virus described herein may be used in diagnostic immunoassays, passive immunotherapy, and generation of antiidiotypic antibodies. The antibodies before being used in passive immunotherapy may be modified, *e.g.*, the antibodies may be chimerized or humanized. See, *e.g.*, U.S. Patent Nos. 4,444,887 and 4,716,111; and International Publication Nos. WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741 for reviews on the generation of chimeric and humanized antibodies. In addition, the ability of the antibodies to neutralize influenza virus and the specificity of the antibodies for influenza virus antigens may be tested prior to using the antibodies in passive immunotherapy. See Section 5.7, *infra* for a discussion regarding use of neutralizing antibodies for the prevention and/or treatment of an influenza virus infection and the disease caused by an influenza virus infection.

[0224] The antibodies elicited or identified using a recombinant influenza virus described herein may be incorporated into compositions. In a specific case, the compositions are pharmaceutical compositions. In some cases, a pharmaceutical composition may comprise one or more other therapies in addition to an antibody. The pharmaceutical compositions can be in any form that allows for the composition to be administered to a subject. In a specific case, the pharmaceutical compositions are suitable for veterinary and/or human administration. In another specific case, the antibody compositions are formulated for the intended route of administration (*e.g.*, parenteral, intransal, or pulmonary administration). The antibody compositions may be used in methods of preventing and/or treating an influenza virus infection. The antibody compositions may also be used in methods or preventing and/or treating influenza virus disease.

[0225] Antibodies elicited or identified using a recombinant influenza virus described herein may be used to monitor the efficacy of a therapy and/or disease progression. Any immunoassay system known in the art may be used for this purpose including, but not limited to, competitive and noncompetitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assays), "sandwich" immunoassays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays and immunoelectrophoresis assays, to name but a few.

[0226] Antibodies elicited or identified using a recombinant influenza virus described herein may be used in the pro-

duction of antiidiotypic antibody. The antiidiotypic antibody can then in turn be used for immunization, in order to produce a subpopulation of antibodies that bind a particular antigen of influenza, *e.g.*, a neutralizing epitope of a hemagglutinin polypeptide (Jerne, 1974, Ann. Immunol. (Paris) 125c:373; Jerne et al., 1982, EMBO J. 1:234.

5.7 PROPHYLACTIC AND THERAPEUTIC USES

[0227] In one aspect, provided herein are methods for inducing an immune response in a subject utilizing a recombinant influenza virus provided herein or an immunogenic composition thereof. In a specific embodiment, a method for inducing an immune response to an influenza virus in a subject comprises administering to a subject in need thereof an effective amount of a recombinant influenza virus or an immunogenic composition thereof. In certain embodiments, the recombinant influenza virus or immunogenic composition thereof expresses influenza virus proteins from two or more types, subtypes, or strains of influenza virus, and thus, may be used to induce an immune response to two or more types, subtypes, or strains of influenza virus. In a specific embodiment, a method for inducing an immune response to an influenza virus in a subject comprises administering to a subject in need thereof a recombinant influenza virus provided herein as a live virus vaccine. In particular embodiments, the live virus vaccine comprises an attenuated virus. In another embodiment, a method for inducing an immune response to an influenza virus in a subject comprises administering to a subject in need thereof a recombinant influenza virus provided herein as an inactivated virus vaccine.

[0228] In a specific embodiment, a method for inducing an immune response in a subject comprises administering to the subject a recombinant influenza virus provided herein which contains nine gene segments, or an immunogenic composition thereof. In certain embodiments, the nine segmented recombinant influenza virus encodes and/or expresses influenza virus antigens from two different types, subtypes, or strains of influenza virus. In a specific embodiment, the nine segmented recombinant influenza virus encodes and/or expresses HA antigens from two different types, subtypes, or strains of influenza virus. In some embodiments, the nine segmented recombinant influenza virus encodes and/or expresses influenza virus antigens and at least one, two, three, or four or 1 to 3, 1 to 4, or 2 to 4 non-influenza virus antigens.

[0229] In another aspect, provided herein are methods for preventing and/or treating an influenza virus infection in a subject utilizing a recombinant influenza virus provided herein or a pharmaceutical composition thereof. In one embodiment, a method for preventing or treating an influenza virus infection in a subject comprises administering to a subject in need thereof an effective amount of a recombinant influenza virus or a composition thereof. In another embodiment, a method for preventing or treating an influenza virus infection in a subject comprises administering to a subject in need thereof an effective amount of a recombinant influenza virus or a pharmaceutical composition thereof and one or more other therapies. In another embodiment, a method for preventing or treating an influenza virus infection in a subject comprises administering to a subject in need thereof a recombinant influenza virus provided herein as a live virus vaccine. In particular embodiments, the live virus vaccine comprises an attenuated virus. In another embodiment, a method for preventing or treating an influenza virus infection in a subject comprises administering to a subject in need thereof a recombinant influenza virus provided herein as an inactivated virus vaccine.

[0230] In a specific embodiment, a method for preventing or treating an influenza virus infection in a subject comprises administering to a subject in need thereof a recombinant influenza virus provided herein which contains nine gene segments, or a pharmaceutical composition thereof. In certain embodiments, the nine segmented recombinant influenza virus encodes and/or expresses influenza virus antigens from two different types, subtypes, or strains of influenza virus. In a specific embodiment, the nine segmented recombinant influenza virus encodes and/or expresses HA antigens from two different types, subtypes, or strains of influenza virus. In some embodiments, the nine segmented recombinant influenza virus encodes and/or expresses influenza virus antigens and at least one, two, three, or four or 1 to 3, 1 to 4, or 2 to 4 non-influenza virus antigens.

[0231] In another aspect, provided herein are methods for preventing and/or treating an influenza virus disease in a subject utilizing a recombinant influenza virus provided herein or a pharmaceutical composition thereof. In a specific embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof an effective amount of a recombinant influenza virus or a pharmaceutical composition thereof. In another embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof an effective amount of a recombinant influenza virus or a pharmaceutical composition thereof and one or more other therapies. In another embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof a recombinant influenza virus provided herein as a live virus vaccine. In particular embodiments, the live virus vaccine comprises an attenuated virus. In another embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof a recombinant influenza virus provided herein as an inactivated virus vaccine.

[0232] In a specific embodiment, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof a recombinant influenza virus provided herein which contains nine gene segments, or a pharmaceutical composition thereof. In certain embodiments, the nine segmented recombinant influenza virus encodes and/or expresses influenza virus antigens from two different types, subtypes, or strains of influenza virus.

In a specific embodiment, the nine segmented recombinant influenza virus encodes and/or expresses HA antigens from two different types, subtypes, or strains of influenza virus. In some embodiments, the nine segmented recombinant influenza virus encodes and/or expresses influenza virus antigens and at least one, two, three, or four or 1 to 3, 1 to 4, or 2 to 4 non-influenza virus antigens.

[0233] In another aspect, a recombinant influenza virus provided herein may be used as a delivery vector. In a specific embodiment, a recombinant influenza virus provided herein that expresses a protein heterologous to influenza virus may be used as a vector to deliver the protein to a subject. For example, a recombinant influenza virus provided herein may express a cytokine or growth factor which is beneficial to a subject. In another specific embodiment, a recombinant influenza virus provided herein that expresses an antigen heterologous to influenza virus may be used as a vector to deliver the antigen to a subject to induce an immune response to the antigen. In some embodiments, the antigen is derived from an infectious pathogen, such as a non-influenza virus antigen, a bacterial antigen, a fungal antigen, or a parasitic antigen. In certain embodiments, the antigen is a tumor antigen or a tumor-associated antigen. In some embodiments, the antigen is derived or obtained from a respiratory pathogen (e.g., RSV). Recombinant influenza viruses provided herein that express influenza virus antigens and one or more antigens heterolgous to influenza virus may induce an immune response to influenza virus and the heterologous antigen(s).

[0234] In a specific embodiment, a recombinant influenza virus provided herein which contains nine gene segments is used as a delivery vector. In certain embodiments, the nine segmented recombinant influenza virus encodes and/or expresses influenza virus antigens from two different types, subtypes, or strains of influenza virus. In a specific embodiment, the nine segmented recombinant influenza virus encodes and/or expresses HA antigens from two different types, subtypes, or strains of influenza virus. In some embodiments, the nine segmented recombinant influenza virus encodes and/or expresses influenza virus antigens and at least one, two, three, or four or 1 to 3, 1 to 4, or 2 to 4 non-influenza virus antigens.

[0235] Described herein are methods of preventing and/or treating an influenza virus infection in a subject by administering neutralizing antibodies described herein. In a specific case, a method for preventing or treating an influenza virus infection in a subject comprises administering to a subject in need thereof an effective amount of a neutralizing antibody described herein, or a pharmaceutical composition thereof. In another case, a method for preventing or treating an influenza virus infection in a subject comprises administering to a subject in need thereof an effective amount of a neutralizing antibody described herein, or a pharmaceutical composition thereof and one or more other therapies. In particular cases, the neutralizing antibody is a monoclonal antibody.

[0236] Described herein are methods of preventing and/or treating an influenza virus disease in a subject by administering neutralizing antibodies described herein. In a specific case, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof an effective amount of a neutralizing antibody described herein, or a pharmaceutical composition thereof. In another case, a method for preventing or treating an influenza virus disease in a subject comprises administering to a subject in need thereof an effective amount of a neutralizing antibody described herein, or a pharmaceutical composition thereof and one or more other therapies. In particular cases, the neutralizing antibody is a monoclonal antibody.

[0237] A recombinant influenza virus provided herein or a neutralizing antibody described herein may be administered alone or in combination with another/other type of therapy known in the art to reduce influenza virus infection, to reduce titers of influenza virus in a subject, to reduce the spread of influenza virus between subjects, to inhibit influenza virus replication, to inhibit influenza virus-induced fusion, to reduce the number and/or frequency of symptoms, and/or to inhibit binding of influenza virus to its host cell receptor.

[0238] In a specific embodiment, administration of a recombinant influenza virus provided herein or reduces influenza virus replication by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 45%, at least 35%, at least 30%, at least 25%, at least 20%, or at least 10% relative to replication of Influenza virus in the absence of said recombinant influenza virus or in the presence of a negative control (e.g., an influenza virus that is not a recombinant influenza virus described herein (e.g., a wild-type influenza virus) virus)) in an assay known to one of skill in the art or described herein. Inhibition of influenza virus replication can be determined by detecting the Influenza virus titer in a biological specimens from a subject using methods known in the art (*e.g.*, Northern blot analysis, RT-PCR, Western Blot analysis, *etc.*).

[0239] In a specific embodiment, administration of a recombinant influenza virus provided herein described herein results in reduction of about 1-fold, about 1.5-fold, about 2-fold, about 3-fold, about 4-fold, about 5-fold, about 8-fold, about 10-fold, about 15-fold, about 20-fold, about 25-fold, about 30-fold, about 35-fold, about 40-fold, about 45-fold, about 50-fold, about 55-fold, about 60-fold, about 65-fold, about 70-fold, about 75-fold, about 80-fold, about 85-fold, about 90-fold, about 95-fold, about 100-fold, about 105 fold, about 110-fold, about 115-fold, about 120 fold, about 125-fold or higher in Influenza virus titer in the subject. The fold-reduction in Influenza virus titer may be as compared to a negative control (e.g., an influenza virus that is not a recombinant influenza virus described herein (e.g., a wild-type influenza virus) , as compared to another treatment in a patient or patient population, or as compared to the titer in the patient prior to recombinant virus administration.

[0240] In a specific embodiment, administration of a recombinant influenza virus provided herein results in a reduction of approximately 1 log or more, approximately 2 logs or more, approximately 3 logs or more, approximately 4 logs or more, approximately 5 logs or more, approximately 6 logs or more, approximately 7 logs or more, approximately 8 logs or more, approximately 9 logs or more, approximately 10 logs or more, 1 to 5 logs, 2 to 10 logs, 2 to 5 logs, or 2 to 10 logs in Influenza virus titer in the subject. The log-reduction in Influenza virus titer may be as compared to a negative control (e.g., an influenza virus that is not a recombinant influenza virus described herein (e.g., a wild-type influenza virus) , as compared to another treatment, or as compared to the titer in the patient prior to administration of the recombinant influenza virus.

[0241] In a specific embodiment, administration of a recombinant influenza virus provided herein inhibits or reduces Influenza virus infection of a subject by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 45%, at least 35%, at least 30%, at least 25%, at least 20%, or at least 10% relative to Influenza virus infection of a subject in the absence of said recombinant influenza virus or in the presence of a negative control (e.g., an influenza virus that is not a recombinant influenza virus described herein (e.g., a wild-type influenza virus)) in an assay known to one of skill in the art or described herein.

[0242] In a specific embodiment, administration of a recombinant influenza virus provided herein or a neutralizing antibody described herein inhibits or reduces the spread of Influenza virus in a subject by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 45%, at least 35%, at least 30%, at least 25%, at least 20%, or at least 10% relative to the spread of Influenza virus in a subject in the absence of said recombinant influenza virus or in the presence of a negative control (e.g., an influenza virus that is not a recombinant influenza virus described herein (e.g., a wild-type influenza virus)) in an assay known to one of skill in the art or described herein.

[0243] In a specific embodiment, administration of a recombinant influenza virus provided herein inhibits or reduces the spread of Influenza virus between a subject and at least one other subject by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 45%, at least 35%, at least 30%, at least 25%, at least 20%, or at least 10% relative to the spread of Influenza virus between a subject and at least one other subject in the absence of said recombinant influenza virus or in the presence of a negative control (e.g., an influenza virus that is not a recombinant influenza virus described herein (e.g., a wild-type influenza virus) or a control antibody (e.g., an antibody that does not bind influenza virus)) in an assay known to one of skill in the art or described herein.

[0244] In a specific embodiment, administration of a recombinant influenza virus provided herein reduces the number of and/or the frequency of symptoms of Influenza virus disease or infection in a subject (exemplary symptoms of influenza virus disease include, but are not limited to, body aches (especially joints and throat), fever, nausea, headaches, irritated eyes, fatigue, sore throat, reddened eyes or skin, and abdominal pain).

[0245] In a specific embodiment, administration of a recombinant influenza virus provided herein reduces the incidence of hospitalization by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 45%, at least 35%, at least 30%, at least 25%, at least 20%, or at least 10% relative to the incidence of hospitalization in the absence of administration of said recombinant influenza virus or antibody.

[0246] In a specific embodiment, administration of a recombinant influenza virus provided herein reduces mortality by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 45%, at least 35%, at least 30%, at least 25%, at least 20%, or at least 10% relative to the mortality in the absence of administration of said recombinant influenza virus

[0247] In a specific case, administration of a neutralizing antibody described herein prevents or inhibits influenza virus from binding to its host cell receptor by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 45%, at least 35%, at least 30%, at least 25%, at least 20%, or at least 10% relative to Influenza virus binding to its host cell receptor in the absence of said antibody(ies) or in the presence of a negative control (e.g., a control antibody (e.g., an antibody that does not bind influenza virus)) in an assay known to one of skill in the art or described herein.

[0248] In a specific case, administration of a neutralizing antibody described herein prevents or inhibits influenza virus-induced fusion by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 45%, at least 35%, at least 30%, at least 25%, at least 20%, or at least 10% relative to Influenza virus -induced fusion in the absence of said antibody(ies) or in the presence of a negative control (e.g., a control antibody (e.g., an antibody that does not bind influenza virus)) in an assay known to one of skill in the art or described herein.

[0249] In a specific case, administration of a neutralizing antibody described herein prevents or inhibits influenza virus-induced fusion after viral attachment to cells by at least 99%, at least 95%, at least 90%, at least 85%, at least 80%, at least 75%, at least 70%, at least 60%, at least 50%, at least 45%, at least 40%, at least 45%, at least 35%, at least 30%, at least 25%, at least 20%, or at least 10% relative to Influenza virus-induced fusion after viral attachment to cells in the

absence of said antibody(ies) or in the presence of a negative control (e.g., a control antibody (e.g., an antibody that does not bind influenza virus))in an assay known to one of skill in the art or described herein.

[0250] In accordance with the methods encompassed herein, a recombinant influenza virus provided herein or antibody described herein or generated in accordance with the methods provided herein may be used as any line of therapy, including, but not limited to, a first, second, third, fourth and/or fifth line of therapy. Further, in accordance with the methods encompassed herein, a recombinant influenza virus provided herein or antibody described herein or generated in accordance with the methods provided herein can be used before or after any adverse effects or intolerance of the therapies other than a recombinant influenza virus or antibody described herein or generated in accordance with the methods provided herein occurs. Encompassed herein are methods for administering one or more recombinant influenza viruses provided herein and/or antibodies described herein or generated in accordance with the methods provided herein to prevent the onset of an Influenza virus disease and/or to treat or lessen the recurrence of an Influenza virus disease.

5.7.1. PATIENT POPULATION

[0251] In one embodiment, a patient treated or prevented in accordance with the methods provided herein is a naive subject, *i.e.*, a subject that does not have a disease caused by influenza virus infection or has not been and is not currently infected with an influenza virus infection. In another embodiment, a patient treated or prevented in accordance with the methods provided herein is a naive subject that is at risk of acquiring an influenza virus infection. In another embodiment, a patient treated or prevented in accordance with the methods provided herein is a patient suffering from or expected to suffer from an influenza virus disease. In another embodiment, a patient treated or prevented in accordance with the methods provided herein is a patient diagnosed with an influenza virus infection or a disease associated therewith. In some embodiments, a patient treated or prevented in accordance with the methods provided herein is a patient infected with an influenza virus that does not manifest any symptoms of influenza virus disease.

[0252] In another embodiment, a patient treated or prevented in accordance with the methods provided herein is a patient experiencing one or more symptoms of influenza virus disease. Symptoms of influenza virus disease include, but are not limited to, body aches (especially joints and throat), fever, nausea, headaches, irritated eyes, fatigue, sore throat, reddened eyes or skin, and abdominal pain. In another embodiment, a patient treated or prevented in accordance with the methods provided herein is a patient with influenza virus disease who does not manifest symptoms of the disease that are severe enough to require hospitalization.

[0253] In another embodiment, a patient treated or prevented in accordance with the methods provided herein is a patient infected with an influenza A virus, an influenza B virus or influenza C virus. In another embodiment, a patient treated or prevented in accordance with the methods provided herein is a patient infected with a particular subtype of influenza A virus. In another embodiment, a patient treated or prevented in accordance with the methods provided herein is a patient infected with an H1 or H3 subtype influenza A virus. In accordance with such embodiments, the patients that are infected with the virus may manifest symptoms of influenza virus disease.

[0254] In some embodiments, a subject to be administered an active compound or composition described herein is an animal. In certain embodiments, the animal is a bird. In certain embodiments, the animal is a canine. In certain embodiments, the animal is a feline. In certain embodiments, the animal is a horse. In certain embodiments, the animal is a cow. In certain embodiments, the animal is a mammal, *e.g.*, a horse, swine, mouse, or primate, preferably a human.

[0255] In a specific embodiment, a patient treated or prevented in accordance with the methods provided herein is a human. In certain embodiments, a patient treated or prevented in accordance with the methods provided herein is a human infant. In some embodiments, a patient treated or prevented in accordance with the methods provided herein is a human toddler. In certain embodiments, a patient treated or prevented in accordance with the methods provided herein is a human child. In other embodiments, a patient treated or prevented in accordance with the methods provided herein is a human adult. In some embodiments, a patient treated or prevented in accordance with the methods provided herein is an elderly human.

[0256] In specific embodiments, a patient treated or prevented in accordance with the methods provided herein is any infant or child more than 6 months of age and any adult over 50 years of age. In other embodiments, the subject is an individual who is pregnant. In another embodiment, the subject is an individual who may or will be pregnant during the influenza season (*e.g.*, November to April in the Northern hemisphere). In specific embodiments, a patient treated or prevented in accordance with the methods provided herein is a woman who has given birth 1, 2, 3, 4, 5, 6, 7, or 8 weeks earlier.

[0257] In some embodiments, a patient treated or prevented in accordance with the methods provided herein is any subject at increased risk of influenza virus infection or disease resulting from influenza virus infection (*e.g.*, an immunocompromised or immunodeficient individual). In some embodiments, a patient treated or prevented in accordance with the methods provided herein is any subject in close contact with an individual with increased risk of influenza virus infection or disease resulting from influenza virus infection (*e.g.*, immunocompromised or immunosuppressed individuals).

[0258] In some embodiments, a patient treated or prevented in accordance with the methods provided herein is a subject affected by any condition that increases susceptibility to influenza virus infection or complications or disease resulting from influenza virus infection. In other embodiments, a patient treated or prevented in accordance with the methods provided herein is a subject in which an influenza virus infection has the potential to increase complications of another condition that the individual is affected by, or for which they are at risk. In particular embodiments, such conditions that increase susceptibility to influenza virus complications or for which influenza virus increases complications associated with the condition are, *e.g.*, conditions that affect the lung, such as cystic fibrosis, emphysema, asthma, or bacterial infections (e.g., infections caused by *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Legionella pneumophila*, and *Chlamydia trachomatus*); cardiovascular disease (e.g., congenital heart disease, congestive heart failure, and coronary artery disease); endocrine disorders (e.g., diabetes); and neurological and neuron-developmental conditions (e.g., disorders of the brain, the spinal cord, the peripheral nerve, and muscle (such as cerebral palsy, epilepsy (seizure disorders), stroke, intellectual disability (e,g, mental retardation), muscular dystrophy, and spinal cord injury)). Other conditions that may increase influenza virus complications include kidney disorders; blood disorders (including anemia or sickle cell disease); or weakened immune systems (including immunosuppression caused by medications, malignancies such as cancer, organ transplant, or HIV infection).

[0259] In some embodiments, a patient treated or prevented in accordance with the methods provided herein is a subject that resides in a group home, such as a nursing home or orphanage. In some embodiments, a patient treated or prevented in accordance with the methods provided herein is subject that works in, or spends a significant amount of time in, a group home, *e.g.*, a nursing home or orphanage. In some embodiments, a patient treated or prevented in accordance with the methods provided herein is a health care worker (*e.g.*, a doctor or nurse). In some embodiments, a patient treated or prevented in accordance with the methods provided herein resides in a dormitory (e.g., a college dormitory). In some embodiments, a patient treated or prevented in accordance with the methods provided herein is a member of the military. In some embodiments, a patient treated or prevented in accordance with the methods provided herein is a child that attends school.

[0260] In some embodiments, a patient treated or prevented in accordance with the methods provided herein is a subject at increased risk of developing complications from influenza virus infection including: any individual who can transmit influenza viruses to those at high risk for complications, such as, *e.g.*, members of households with high-risk individuals, including households that will include infants younger than 6 months, individuals coming into contact with infants less than 6 months of age, or individuals who will come into contact with individuals who live in nursing homes or other long-term care facilities; individuals with long-term disorders of the lungs, heart, or circulation; individuals with metabolic diseases (*e.g.*, diabetes); individuals with kidney disorders; individuals with blood disorders (including anemia or sickle cell disease); individuals with weakened immune systems (including immunosuppression caused by medications, malignancies such as cancer, organ transplant, or HIV infection); and children who receive long-term aspirin therapy (and therefore have a higher chance of developing Reye syndrome if infected with influenza).

[0261] In other embodiments, a patient treated or prevented in accordance with the methods provided herein includes healthy individuals six months of age or older, who: plan to travel to foreign countries and areas where flu outbreaks may be occurring, such, *e.g.*, as the tropics and the Southern Hemisphere from April through September; travel as a part of large organized tourist groups that may include persons from areas of the world where influenza viruses are circulating; attend school or college and reside in dormitories, or reside in institutional settings; or wish to reduce their risk of becoming ill with influenza virus disease.

[0262] In specific embodiments, a patient treated or prevented in accordance with the methods provided herein is an individual who is susceptible to adverse reactions to conventional therapies. In other embodiments, the patient may be a person who has proven refractory to therapies other than a recombinant influenza virus or antibody described herein but are no longer on these therapies. In certain embodiments, a patient with an influenza virus disease is refractory to a therapy when the infection has not significantly been eradicated and/or the symptoms have not been significantly alleviated. The determination of whether a patient is refractory can be made either in vivo or in vitro by any method known in the art for assaying the effectiveness of a therapy for infections, using art-accepted meanings of "refractory" in such a context. In various embodiments, a patient with an influenza virus disease is refractory when viral replication has not decreased or has increased following therapy.

[0263] In certain embodiments, patients treated or prevented in accordance with the methods provided herein are patients already being treated with antibiotics, anti-virals, antifungals, or other biological therapy/immunotherapy. Among these patients are refractory patients, patients who are too young for conventional therapies, and patients with reoccurring influenza virus disease or a symptom relating thereto despite treatment with existing therapies.

[0264] In certain embodiments, patients receving a recombinant influenza virus described herein that expresses a protein heterologous to influenza virus are patients that may benefit from the expression of such a protein. For example, if the heterologous protein is a cytokine or growth factor and the patient has a condition or disease, the expression of the cytokine or growth factor may beneficial for the treatment of the condition or disease.

[0265] In certain embodiments, patients receiving a recombinant influenza virus described herein that expresses an

antigen heterologous to influenza virus are patients that are infected or susceptible to infection with the pathogen from which the heterolgous antigen is derived. In some embodiments, patients receiving a recombinant influenza virus described herein that expresses an antigen heterologous to influenza virus are patients that are diagnosed with an infection with the pathogen from which the heterologous antigen is derived. In some embodiments, patients receiving a recombinant influenza virus described herein that expresses an antigen heterologous to influenza virus are patients manifest one or more symptoms of a disease associated with an infection with the pathogen from which the heterologous antigen is derived. In some embodiments, patients receiving a recombinant influenza virus described herein that expresses an antigen heterologous to influenza virus are patients that are diagnosed with a disease associated with an infection with the pathogen from which the heterologous antigen is derived. In some embodiments, the antigen is from a respiratory pathogen, e.g., the antigen is or is derived from the F, G, or M2 protein of RSV, the spike protein of a Coronavirus (e.g., SARS, HuCoV), the F protein of human metapneumovirus, the F or HN protein of parainfluenza virus, the G or F protein of Hendra virus, the G or F protein of Nipah virus, or the capsid protein of Adenovirus.

[0266] In certain embodiments, patients receiving a recombinant influenza virus described herein that expresses a tumor antigen or tumor associated antigen are patients with cancer, susceptible to cancer or at risk of getting cancer. In some embodiments, patients receiving a recombinant influenza virus described herein that expresses a tumor antigen or tumor associated antigen are patients with a genetic predisposition for cancer. In certain embodiments, patients receiving a recombinant influenza virus described herein that expresses a tumor antigen or tumor associated antigen are patients with diagnosed with cancer. In specific embodiments, the tumor antigen or tumor associated antigen expressed by a recombinant influenza virus makes sense with respect to the cancer being treated. For example, if a subject has lung cancer, a recombinant influenza virus that expresses an antigen associated with the lung cancer is administered the subject. In a specific embodiment, the cancer is a solid tumor cancer, such as, *e.g.*, a sarcoma, melanoma, lymphoma and carcinoma. In another embodiment, the cancer is a non-solid cancer, such as leukemia. Non-limiting examples of cancers include brain cancer, lung cancer, colon cancer, pancreatic cancer, liver cancer, skin cancer, breast cancer, prostate cancer, bone cancer, and uterine cancer.

[0267] In some embodiments, it may be advisable not to administer a live virus vaccine to one or more of the following patient populations: elderly humans; infants younger than 6 months old; pregnant individuals; infants under the age of 1 years old; children under the age of 2 years old; children under the age of 3 years old; children under the age of 4 years old; children under the age of 5 years old; adults under the age of 20 years old; adults under the age of 25 years old; adults under the age of 30 years old; adults under the age of 35 years old; adults under the age of 40 years old; adults under the age of 45 years old; adults under the age of 50 years old; elderly humans over the age of 70 years old; elderly humans over the age of 75 years old; elderly humans over the age of 80 years old; elderly humans over the age of 85 years old; elderly humans over the age of 90 years old; elderly humans over the age of 95 years old; children and adolescents (2-17 years of age) receiving aspirin or aspirin-containing medications, because of the complications associated with aspirin and wild-type influenza virus infections in this age group; individuals with a history of asthma or other reactive airway diseases; individuals with chronic underlying medical conditions that may predispose them to severe influenza infections; individuals with a history of Guillain-Barre syndrome; individuals with acute serious illness with fever; or individuals who are moderately or severely ill. For such individuals, administration of inactivated virus vaccines, split virus vaccines, subunit vaccines, virosomes, viral-like particles or the non-viral vectors described herein may be preferred. In certain embodiments, subjects preferably administered a live virus vaccine may include healthy children and adolescents, ages 2-17 years, and healthy adults, ages 18-49.

[0268] In certain embodiments, an immunogenic formulation comprising a live virus is not given concurrently with other live-virus vaccines.

5.7.2. DOSAGE & FREQUENCY OF ADMINISTRATION

[0269] A recombinant influenza virus, or a composition provided herein may be delivered to a subject by a variety of routes. These include, but are not limited to, intranasal, intratracheal, oral, intradermal, intramuscular, topical intraperitoneal, transdermal, intravenous, pulmonary, conjunctival and subcutaneous routes. In some embodiments, a composition is formulated for topical administration, for example, for application to the skin. In specific embodiments, the composition is formulated for nasal administration, *e.g.*, as part of a nasal spray. In certain embodiments, a composition is formulated for intramuscular administration. In some embodiments, a composition is formulated for subcutaneous administration. In specific embodiments for live virus vaccines, the vaccine is formulated for administration by a route other than injection.

[0270] When a recombinant influenza virus is to be administered to a subject, it may be preferable to introduce an immunogenic composition via the natural route of infection of influenza virus. The ability of a recombinant influenza virus to induce a vigorous secretory and cellular immune response can be used advantageously. For example, infection of the respiratory tract by a recombinant influenza virus may induce a strong secretory immune response, for example in the urogenital system, with concomitant protection against an influenza virus. In addition, in a preferred embodiment it

may be desirable to introduce the pharmaceutical compositions into the lungs by any suitable route. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent for use as a spray.

[0271] In some embodiments, when a recombinant influenza virus or a composition thereof is administered to a non-human subject (*e.g.*, a non-human subject), the virus or composition is administered orally to the subject in the subject's food. In other embodiments, when a recombinant influenza virus or a composition thereof is administered to a subject (*e.g.*, a non-human subject), the virus or composition is administered orally to the subject in the subject's water. In other embodiments, when a recombinant influenza virus or a composition thereof is administered to a non-human subject, the virus or composition is administered by spraying the subject with the virus or composition.

[0272] The amount of a recombinant influenza virus, or composition provided herein which will be effective in the treatment and/or prevention of an influenza virus infection or an influenza virus disease will depend on the nature of the disease, and can be determined by standard clinical techniques. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the infection or disease caused by it, and should be decided according to the judgment of the practitioner and each subject's circumstances. For example, effective doses may also vary depending upon means of administration, target site, physiological state of the patient (including age, body weight, health), whether the patient is human or an animal, whether other medications are administered, and whether treatment is prophylactic or therapeutic. Similarly, the amount of a recombinant influenza virus or a composition thereof that will be effective as a delivery vector will vary and can be determined by standard clinical techniques. Treatment dosages are optimally titrated to optimize safety and efficacy.

[0273] In certain embodiments, an *in vitro* assay is employed to help identify optimal dosage ranges. Effective doses may be extrapolated from dose response curves derived from *in vitro* or animal model test systems.

[0274] Exemplary doses for live recombinant influenza virus may vary from 10-100, or more, virions per dose. In some embodiments, suitable dosages of a live recombinant influenza virus are 10^2, 5×10^2, 10^3, 5×10^3, 10^4 5×10^4, 10^5 5×10^5 10^6, 5×10^6, 10^7, 5×10^7, 10^8, 5×10^8, 1×10^9, 5×10^9, 1×10^{10}, 5×10^{10}, 1×10^{11}, 5×10^{11} or 10^{12} pfu, and can be administered to a subject once, twice, three or more times with intervals as often as needed. In another embodiment, a live recombinant influenza virus is formulated such that a 0.2-mL dose contains $10^{6.5\text{-}7.5}$ fluorescent focal units of live recombinant influenza viruses. In another embodiment, an inactivated vaccine is formulated such that it contains about 15 μg to about 100 μg, about 15 μg to about 75 μg, about 15 μg to about 50 μg, or about 15 μg to about 30 μg of an influenza hemagglutinin.

[0275] In certain embodiments, a recombinant influenza virus provided herein or a composition thereof is administered to a subject as a single dose followed by a second dose 3 to 6 weeks later. In accordance with these embodiments, booster inoculations may be administered to the subject at 6 to 12 month intervals following the second inoculation. In certain embodiments, the booster inoculations may utilize a different recombinant influenza virus or a composition thereof. In some embodiments, the administration of the same recombinant influenza virus or a composition thereof may be repeated and the administrations may be separated by at least 1 day, 2 days, 3 days, 5 days, 10 days, 15 days, 30 days, 45 days, 2 months, 75 days, 3 months, or at least 6 months.

[0276] In specific embodiments for administration to children, two doses of a recombinant influenza virus described herein or a composition thereof, given at least one month apart, are administered to a child. In specific embodiments for administration to adults, a single dose of a recombinant influenza virus described herein or a composition thereof is given. In another embodiment, two doses of a recombinant influenza virus described herein or a composition thereof, given at least one month apart, are administered to an adult. In another embodiment, a young child (six months to nine years old) may be administered a recombinant influenza virus described herein or a composition thereof for the first time in two doses given one month apart. In a particular embodiment, a child who received only one dose in their first year of vaccination should receive two doses in the following year. In some embodiments, two doses administered 4 weeks apart are preferred for children 2 -8 years of age who are administered an immunogenic composition described herein, for the first time. In certain embodiments, for children 6-35 months of age, a half dose (0.25 ml) may be preferred, in contrast to 0.5 ml which may be preferred for subjects over three years of age.

[0277] In particular embodiments, a recombinant influenza virus or a composition thereof is administered to a subject in the fall or winter, *i.e.*, prior to or during the influenza season in each hemisphere. In one embodiment, children are administered their first dose early in the season, *e.g.*, late September or early October for the Northern hemisphere, so that the second dose can be given prior to the peak of the influenza season.

[0278] For passive immunization with an antibody, the dosage ranges from about 0.0001 to 100 mg/kg, and more usually 0.01 to 50 mg/kg or 0.1 to 15 mg/kg, of the patient body weight. For example, dosages can be 1 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg or in other words, 70 mg or 700 mg or within the range of 70-700 mg, respectively, for a 70 kg patient. An exemplary treatment regime entails administration once per every two weeks or once a month or once every 3 to 6 months for a period of one year or over several years, or over several year-intervals. In some methods, two or more monoclonal antibodies with different binding specificities are administered simultaneously, in which case the dosage of each antibody administered falls within the ranges indicated. Antibody is

usually administered on multiple occasions. Intervals between single dosages can be weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of antibody to the recombinant influenza virus in the patient.

5.7.3. ADDITIONAL THERAPIES

[0279] In various embodiments, a recombinant influenza virus provided herein may be administered to a subject in combination with one or more other therapies (*e.g.*, antiviral or immunomodulatory therapies). In some embodiments, a pharmaceutical composition provided herein may be administered to a subject in combination with one or more therapies. The one or more other therapies may be beneficial in the treatment or prevention of an influenza virus disease or may ameliorate a condition associated with an influenza virus disease.

[0280] In some embodiments, the one or more other therapies that are supportive measures, such as pain relievers, anti-fever medications, or therapies that alleviate or assist with breathing. Specific examples of supportive measures include humidification of the air by an ultrasonic nebulizer, aerolized racemic epinephrine, oral dexamethasone, intravenous fluids, intubation, fever reducers (*e.g.*, ibuprofen, acetometaphin), and antibiotic and/or antifungal therapy (*i.e.*, to prevent or treat secondary bacterial and/or fungal infections).

[0281] In certain embodiments, the therapies are administered less than 5 minutes apart, less than 30 minutes apart, 1 hour apart, at about 1 hour apart, at about 1 to about 2 hours apart, at about 2 hours to about 3 hours apart, at about 3 hours to about 4 hours apart, at about 4 hours to about 5 hours apart, at about 5 hours to about 6 hours apart, at about 6 hours to about 7 hours apart, at about 7 hours to about 8 hours apart, at about 8 hours to about 9 hours apart, at about 9 hours to about 10 hours apart, at about 10 hours to about 11 hours apart, at about 11 hours to about 12 hours apart, at about 12 hours to 18 hours apart, 18 hours to 24 hours apart, 24 hours to 36 hours apart, 36 hours to 48 hours apart, 48 hours to 52 hours apart, 52 hours to 60 hours apart, 60 hours to 72 hours apart, 72 hours to 84 hours apart, 84 hours to 96 hours apart, or 96 hours to 120 hours part. In specific embodiments, two or more therapies are administered within the same patent visit.

[0282] Any anti-viral agents well-known to one of skill in the art may be used in combination with a recombinant influenza virus provided herein or pharmaceutical composition thereof. Non-limiting examples of anti-viral agents include proteins, polypeptides, peptides, fusion proteins antibodies, nucleic acid molecules, organic molecules, inorganic molecules, and small molecules that inhibit and/or reduce the attachment of a virus to its receptor, the internalization of a virus into a cell, the replication of a virus, or release of virus from a cell. In particular, anti-viral agents include, but are not limited to, nucleoside analogs (*e.g.*, zidovudine, acyclovir, gangcyclovir, vidarabine, idoxuridine, trifluridine, and ribavirin), foscarnet, amantadine, rimantadine, saquinavir, indinavir, ritonavir, alpha-interferons and other interferons, AZT, zanamivir (Relenza®), and oseltamavir (Tamiflu®). In certain embodiments, a recombinant influenza virus provided herein, or a pharmaceutical composition provided herein is administered in combination with an influenza virus vaccine, *e.g.*, Fluarix® (GlaxoSmithKline), FluMist® (MedImmune Vaccines), Fluvirin® (Chiron Corporation), Fluzone® (Aventis Pasteur). In specific embodiments, the anti-viral agent is an immunomodulatory agent that is specific for a viral antigen. In particular embodiments, the viral antigen is an influenza virus antigen.

[0283] In a specific embodiment, one or more therapies that prevent or treat secondary responses to a primary influenza virus infection are administered in combination with a recombinant influenza virus provided herein, or a pharmaceutical composition provided herein. Examples of secondary responses to a primary influenza virus infection include, but are not limited to, asthma-like responsiveness to mucosal stimuli, elevated total respiratory resistance, increased susceptibility to secondary viral, bacterial, and fungal infections, and development of conditions such as, but not limited to, bronchiolitis, pneumonia, croup, and febrile bronchitis.

[0284] In some embodiments, a recombinant influenza virus provided herein or a pharmaceutical composition thereof is administered in combination with an antibody that specifically binds to an influenza virus antigen.

5.8 BIOLOGICAL ASSAYS

Reassortment Assays

[0285] A reverse genetics approach can be used to assess whether each of the chimeric gene segments of the recombinant influenza viruses shown in, *e.g.*, Figures 35 to 37, can reassort. Cells expressing the necessary influenza virus proteins can be co-transfected with influenza virus chimeric segments that have had their packaging signals swapped and influenza virus gene segments from a wild-type or lab strain of influenza virus, wherein the wild-type or lab strain influenza virus gene segments include a gene segment that encodes an influenza virus protein encoded by one of the chimeric influenza virus gene segments and the other gene segments necessary to produce a replication-competent influenza virus. For example, cells, such as 293T cells, MDCK cells or Vero cells, expressing the necessary viral proteins (*e.g.*, PA, PB1, PB2, and NP) can be transfected with plasmids encoding four of the chimeric gene segments shown in Figure 35 (NA-PB2mut-NA, PB2-PB1mut-PB2, PB1-PAmut-PB1, and PA-NAmut-PA) and plasmids encoding

five gene segments (pDZ-NP, NA, M, NS, and HA) of a wild-type influenza virus or a lab strain, such as A/PR/8/34, using techniques previously described (see, *e.g.*, Gao et al., 2008, J. Virol. 82: 6419-6426; Quinlivan et al., 2005, J. Virol. 79: 8431-8439; Fodor et al., 1999, J. Virol. 73: 9679-9682). The recombinant viruses rescued can then be grown in tissue culture or embryonated eggs and plaque purified using known techniques. The gene segments present in the plaque purified viruses can then be determined by, *e.g.*, amplifying single plaques, isolating the vRNA from the virus, subjecting the vRNA to RT-PCR using primers designed to hybridize to specific gene segments and running the RT-PCR products on an agarose gel. Alternatively, the vRNA segments from the plaque performed viruses can be sequenced using techniques known in the art, such as deep sequencing. The inability to detect influenza viruses containing less than the combination of the chimeric gene segments that have had their packaging signals swapped indicates that those chimeric gene segments are unable to reassort freely. For example, with respect to the chimeric gene segments of the recombinant virus shown in Figure 35, the inability to detect influenza viruses containing the three chimeric NA-PB2mut-NA, PB2-PB1mut-PB2, and PB1-PAmut-PB1 gene segments and the wild-type or lab strain influenza virus NA, NP, M, NS and HA gene segments indicates that the four chimeric gene segments (NA-PB2mut-NA, PB2-PB1mut-PB2, PB1-PAmut-PB1, and PA-NAmut-PA) are unable to reassort freely.

[0286] As another approach to determine whether the chimeric gene segments of the recombinant influenza viruses shown in, *e.g.*, Figures 35 to 37 can freely reassort in tissue culture, cells (*e.g.*, 293T cells, MDCK cells or Vero cells) can be co-infected with the recombinant virus shown in, *e.g.*, Figure 35, 36 or 37, and a wild-type or lab strain of influenza virus at certain multiplicity of infection ("moi") for each virus (*e.g.*, an moi of 10). The resulting viruses can then be plaque purified. The gene segments present in the plaque purified viruses can then be determined by, *e.g.*, amplifying single plaques, isolating the vRNA from the virus, subjecting the vRNA to RT-PCR using primers designed to hybridize to specific gene segments and running the RT-PCR products on an agarose gel. Alternatively, the vRNA segments from the plaque performed viruses can be sequenced using techniques known in the art, such as deep sequencing. The inability to detect viruses containing less than the combination of the chimeric segments that have had their packaging signals swapped are unable to reassort freely. For example, with respect to the chimeric gene segments of the recombinant virus shown in Figure 35, the inability to detect influenza viruses containing the three chimeric NA-PB2mut-NA, PB2-PB1mut-PB2, and PB1-PAmut-PB1 gene segments and the wild-type or lab strain influenza virus NA, NP, M, NS and HA gene segments indicates that the four chimeric gene segments (NA-PB2mut-NA, PB2-PB1mut-PB2, PB1-PAmut-PB1, and PA-NAmut-PA) are unable to reassort freely.

Assays to Detect the Presence of a Chimeric Influenza Virus Gene Segment

[0287] Any technique known in the art may be used to detect a chimeric influenza virus gene segment or the complement thereof, or a nucleic acid encoding a chimeric influenza virus gene segment. For example, primers may be designed that are specific for a particular chimeric influenza virus gene segment and RT-PCR or PCR using those primers may be performed to amplify a fragment of the segment. The amplified fragment may be detected by, *e.g.*, running the fragment on an agarose gel. Alternatively, primers may be designed that are specific for a particular chimeric influenza virus gene segment and real-time RT-PCR using those primers may be performed. In one case, a pair primers are designed that are specific for a particular chimeric influenza virus gene segment, wherein the one of the primers is a sense primer that anneals to the 3' NCR1 or 3' CRS1 derived from a first type influenza virus gene segment, and the other primer is an antisense primer that anneals to the mORF derived from a second type of influenza virus gene segment. In another embodiment, a pair primers are designed that are specific for a particular chimeric influenza virus gene segment, wherein the one of the primers is an antisense primer that anneals to the 5' NCR1 or 5' CRS1 derived from a first type influenza virus gene segment, and the other primer is a sense primer that anneals to the mORF derived from a second type of influenza virus gene segment. Techniques known to one of skill in the art may be used to design primers that are specific for a particular chimeric influenza virus gene segment.

Packaging Assays

[0288] Incorporation of a chimeric influenza virus gene segment into a virus particle, *i.e.*, packaging, can be assessed by any method known in the art or described herein (*e.g.*, in cell culture, animal model or viral culture in embryonated eggs).

[0289] In one example, viral particles may be purified and RNA isolated and run on a 2.8% denaturing polyacrylamide gel which is then stained with a silver staining kit (Invitrogen) to determine the presence of a chimeric influenza virus gene segment (see, *e.g.*, Gao et al., 2008, J. Virol. 82: 6419-6426 for a description of such an assay).

[0290] In another example, viral particles from cell culture of the allantoic fluid of embryonated eggs can be purified by centrifugation through a sucrose cushion and subsequently analyzed for the presence of a chimeric influenza virus gene segment by RT-PCR.

[0291] Packaging assays can be used to determine the regions of an influenza virus gene segment that are necessary and/or sufficient for packaging. In these cases, a reporter gene can be used to facilitate the assay. Packaging assays

can also be used to determine whether, and if so, to what degree, the chimeric influenza virus gene segments are packaged into a virus particle, wherein the chimeric influenza virus gene segment does not encode a reporter gene.

[0292] Illustrative packaging assays include the packaging assay disclosed in Liang et al., 2005, J Virol 79:10348-10355 and the packaging assay disclosed in Muramoto et al., 2006, J Virol 80:2318-2325. Several parameters of the protocols of Liang and Muramoto can be modified; for example various host cells can be used and various reporter genes can be used.

[0293] In certain cases, the packaging assay of Muramoto et al. is used ("Muramoto protocol"). Briefly, a reporter influenza virus gene segment may be constructed, wherein the reporter gene is flanked by the 3' NCR and the 3' proximal coding region of one type of influenza virus gene segment or a derivative or a fragment thereof, wherein any start codon in the 3' proximal coding region is mutated, on one side and the 5' NCR and the 5' proximal coding region of this type of influenza virus gene segment or derivatives or fragments thereof on the other side. The reporter gene can be GFP. The reporter influenza virus gene segment is transfected with seven plasmids that encode the other seven types of influenza virus gene segments into a host cell, such as 293T cells. In addition, expression plasmids encoding all 10 influenza virus proteins are transfected into the host cell. After virus like particles ("VLPs") are released from the host cell, *e.g.*, after 48 hours, supernatant is collected. The supernatant is then used to infect fresh host cells, *e.g.*, MDCK cells, concurrently with a helper influenza virus. At least one protein of the helper influenza virus is antigenically distinguishable from the same type of protein in the VLP such that cells that are infected with VLP can be identified. The number of cells expressing the reporter gene is determined using, *e.g.*, FACS, and the number of cells expressing VLP protein is determined using immunocytochemistry coupled with FACS. The ratio of reporter gene expressing cells to VLP protein expressing cells is a measure for the efficiency of packaging of the reporter influenza virus gene segment into a virion.

[0294] In certain cases, the packaging assay of Liang et al. is used. Briefly, the eight-plasmid rescue system (Hoffmann et al., 2000, PNAS 97:6208-6113) is combined with a reporter influenza virus gene segment. The reporter influenza virus gene segment is constructed as discussed above for the Muramoto protocol. The eight-plasmid rescue system provides all eight influenza gene segments as plasmids with promoters such that the gene segments can be transcribed in both directions thereby generating all eight wild-type vRNAs and all viral proteins needed for virion production. The eight plasmids and the reporter gene segment are transfected into a host cell, such as 293T cells. After virions are released from the host cell, *e.g.*, after 48 hours, supernatant is collected. Fresh host cells, such as MDBK cells, are infected with the supernatant until the reporter gene is expressed, *e.g.*, for 15 hours. Subsequently, the level of reporter gene expression is tested. An assay suitable for the reporter gene can be selected by the skilled artisan. For example, if the reporter gene is a fluorescent protein, such as GFP, FACS analysis can be used to determine the number of cells that express the reporter gene. The number of cells expressing the reporter gene is representative of the efficiency of packaging, such that a relative low number of cells expressing the reporter gene indicates a low efficiency of packaging of the reporter gene segment and a relative high number of cells expressing the reporter gene indicates a high efficiency of packaging of the reporter gene segment. In certain embodiments, the number of cells expressing the reporter gene is normalized over the cells that produce virus. The number of virus-producing cells can be determined, *e.g.*, by a plaque assay or immunocytochemistry using an antibody against a viral protein, such as NP, paired with FACS analysis.

[0295] The principle of the packaging assays described above with a reporter gene also applies to packaging assays without reporter genes. The skilled artisan could use any known technique to adapt the packaging assays described above to assays without a reporter gene. Instead of relying on detection of the reporter gene product as a read-out of packaging efficiency as described above, the skilled artisan could detect instead either the influenza virus gene segment of interest or the gene product of the influenza virus gene segment of interest. RT-PCR can be used with primers that are specific to the influenza virus gene segment to detect and quantify the influenza virus gene segment of interest. Western blot, ELISA, radioimmunoassay, immunoprecipitation, immunocytochemistry, or immunocytochemistry in conjunction with FACS can be used to quantify the gene product of the influenza virus gene segment of interest as a read-out of packaging efficiency. In is also possible to fuse the gene in the influenza virus gene segment of interest to a sequence that encodes a peptide tag such that the gene product of the gene of the influenza virus gene segment of interest encodes a fusion protein with a peptide tag, wherein the peptide tag can be detected.

Viral Assays

[0296] Viral assays include those that measure viral replication (as determined, *e.g.*, by plaque formation) or the production of viral proteins (as determined, *e.g.*, by western blot analysis) or viral RNAs (as determined, *e.g.*, by RT-PCR or northern blot analysis) in cultured cells *in vitro* using methods which are well known in the art.

[0297] Growth of a recombinant influenza virus provided herein can be assessed by any method known in the art or described herein (*e.g.*, in cell culture (*e.g.*, cultures of chicken embryonic kidney cells or cultures of chicken embryonic fibroblasts (CEF)). Viral titer may be determined by inoculating serial dilutions of a recombinant influenza virus provided herein into cell cultures (*e.g.*, CEF, MDCK, EFK-2 cells, Vero cells, primary human umbilical vein endothelial cells

(HUVEC), H292 human epithelial cell line or HeLa cells), chick embryos, or live animals (*e.g.*, avians). After incubation of the virus for a specified time, the virus is isolated using standard methods. An hemagglutinin (HA) assay may be carried out in V-bottom 96-well plates. Serial twofold dilutions of each sample in PBS are incubated for 1 h on ice with an equal volume of a 0.5% suspension of chicken erythrocytes in PBS. Positive wells contain an adherent, homogeneous layer of erythrocytes; negative wells contain a nonadherent pellet. Physical quantitation of the virus titer can be performed using PCR applied to viral supernatants (Quinn & Trevor, 1997; Morgan et al., 1990), hemagglutination assays, tissue culture infectious doses (TCID50) or egg infectious doses (EID50).

Antibody Assays

[0298] Antibodies generated or identified in accordance with the methods described herein may be characterized in a variety of ways well-known to one of skill in the art (*e.g.*, ELISA, Surface Plasmon resonance display (BIAcore), Western blot, immunofluorescence, immunostaining and/or microneutralization assays). In particular, antibodies generated or identified in accordance may be assayed for the ability to specifically bind to an antigen of the recombinant influenza virus. Such an assay may be performed in solution (*e.g.*, Houghten, 1992, Bio/Techniques 13:412 421), on beads (Lam, 1991, Nature 354:82 84), on chips (Fodor, 1993, Nature 364:555 556), on bacteria (U.S. Patent No. 5,223,409), on spores (U.S. Patent Nos. 5,571,698; 5,403,484; and 5,223,409), on plasmids (Cull et al., 1992, Proc. Natl. Acad. Sci. USA 89:1865 1869) or on phage (Scott and Smith, 1990, Science 249:386 390; Cwirla et al., 1990, Proc. Natl. Acad. Sci. USA 87:6378 6382; and Felici, 1991, J. Mol. Biol. 222:301 310). Antibodies that specifically bind to an antigen of a recombinant influenza virus can then be assayed for their specificity to said antigen.

[0299] Antibodies generated or identified in accordance with the methods described herein may be assayed for specific binding to an antigen of a recombinant virus described herein and cross-reactivity with other antigens by any method known in the art. Immunoassays which can be used to analyze specific binding and cross-reactivity include, but are not limited to, competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (see, *e.g.,* Ausubel et al., eds., 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York,

[0300] The binding affinity of an antibody to an antigen and the off-rate of an antibody-antigen interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled antigen (*e.g.*, ^{3}H or ^{125}I) with the antibody of interest in the presence of increasing amounts of unlabeled antigen, and the detection of the antibody bound to the labeled antigen. The affinity of the antibody can be determined from the data by scatchard plot analysis. Competition with a second antibody can also be determined using radioimmunoassays. In this case, a recombinant virus of described herein or an antigen thereof is incubated with an antibody against the antigen conjugated to a labeled compound (*e.g.*, ^{3}H or ^{125}I) in the presence of increasing amounts of an unlabeled second antibody.

[0301] BIAcore kinetic analysis can be used to determine the binding on and off rates of an antibody to an antigen of a recombinant influenza virus described herein. BIAcore kinetic analysis comprises analyzing the binding and dissociation of polypeptide comprising the antigen of interest from chips with immobilized antibodies generated or identified in accordance with methods described herein on their surface. A typical BIAcore kinetic study involves the injection of 250 μL of an antibody reagent (mAb, Fab) at varying concentration in HBS buffer containing 0.005% Tween-20 over a sensor chip surface, onto which has been immobilized the antigen. The flow rate is maintained constant at 75 μL/min. Dissociation data is collected for 15 min. or longer as necessary. Following each injection/dissociation cycle, the bound mAb is removed from the antigen surface using brief, 1 min. pulses of dilute acid, typically 10-100 mM HCl, though other regenerants are employed as the circumstances warrant. More specifically, for measurement of the rates of association, kon, and dissociation, koff, the polypeptide comprising the antigen is directly immobilized onto the sensor chip surface through the use of standard amine coupling chemistries, namely the EDC/NHS method (EDC= N-diethylaminopropyl)-carbodiimide). Briefly, a 5-100 nM solution of the polypeptide comprising the antigen in 10mM NaOAc, pH4 or pH5 is prepared and passed over the EDC/NHS-activated surface until approximately 30-50 RU's worth of antigen are immobilized. Following this, the unreacted active esters are "capped" off with an injection of 1M Et-NH2. A blank surface, containing no antigen, is prepared under identical immobilization conditions for reference purposes. Once an appropriate surface has been prepared, a suitable dilution series of each one of the antibody reagents is prepared in HBS/Tween-20, and passed over both the antigen and reference cell surfaces, which are connected in series. The range of antibody concentrations that are prepared varies, depending on what the equilibrium binding constant, KD, is estimated to be. As described above, the bound antibody is removed after each injection/dissociation cycle using an appropriate regenerant.

[0302] Antibodies generated or identified in accordance with the methods described herein can also be assayed for

their ability to inhibit the binding of an antigen of a recombinant influenza virus to a host cell using techniques known to those of skill in the art. For example, cells expressing receptors known to bind to influenza virus can be contacted with influenza virus in the presence or absence of an antibody generated or identified in accordance with the methods described herein and the ability of the antibody to inhibit the binding can measured by, for example, flow cytometry or a scintillation assay. The antigen or the antibody can be labeled with a detectable compound such as a radioactive label (*e.g.*, ^{32}P, ^{35}S, and ^{125}I) or a fluorescent label (*e.g.*, fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine) to enable detection of an interaction between the influenza virus and a cell.

Antiviral Activity Assays

[0303] Antibodies described herein or compositions thereof can be assessed *in vitro* for antiviral activity. In one embodiment, the antibodies or compositions thereof are tested *in vitro* for their effect on growth of an influenza virus. Growth of influenza virus can be assessed by any method known in the art or described herein (*e.g.*, in cell culture). In a specific embodiment, cells are infected at a MOI of 0.0005 and 0.001, 0.001 and 0.01, 0.01 and 0.1, 0.1 and 1, or 1 and 10, or a MOI of 0.0005, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5 or 10 and incubated with serum free media supplemented. Viral titers are determined in the supernatant by hemagglutinin plaques or any other viral assay described herein. Cells in which viral titers can be assessed include, but are not limited to, EFK-2 cells, Vero cells, primary human umbilical vein endothelial cells (HUVEC), H292 human epithelial cell line and HeLa cells. *In vitro* assays include those that measure altered viral replication (as determined, *e.g.*, by plaque formation) or the production of viral proteins (as determined, *e.g.*, by Western blot analysis) or viral RNAs (as determined, e.g., by RT-PCR or northern blot analysis) in cultured cells *in vitro* using methods which are well known in the art or described herein.

[0304] In one non-limiting example, a monolayer of the target mammalian cell line is infected with different amounts (*e.g.*, multiplicity of 3 plaque forming units (pfu) or 5 pfu) of influenza and subsequently cultured in the presence or absence of various dilutions of antibodies (*e.g.*, 0.1 μg/ml, 1 μg/ml, 5 μg/ml, or 10 pg/ml). Infected cultures are harvested 48 hours or 72 hours post infection and titered by standard plaque assays known in the art on the appropriate target cell line (*e.g.*, Vero cells).

[0305] In a non-limiting example of a hemagglutination assay, cells are contacted with an antibody and are concurrently or subsequently infected with the virus (*e.g.*, at an MOI of 1) and the virus is incubated under conditions to permit virus replication (*e.g.*, 20-24 hours). The antibodies are preferably present throughout the course of infection. Viral replication and release of viral particles is then determined by hemagglutination assays using 0.5% chicken red blood cells. See, *e.g.*, Kashyap et al., PNAS USA 105: 5986-5991. In some embodiments, an antibody compound is considered an inhibitor of viral replication if it reduces viral replication by at least 2 wells of HA, which equals approximately a 75% reduction in viral titer. In specific embodiments, an inhibitor reduces viral titer in this assay by 50% or more, by 55% or more, by 60% or more, by 65% or more, by 70% or more, by 75% or more, by 80% or more, by 85% or more, by 90% or more, or by 95% or more.

Cytotoxicity Assays

[0306] Many assays well-known in the art can be used to assess viability of cells (infected or uninfected) or cell lines following exposure to a recombinant influenza virus provided herein, an antibody described herein or a composition thereof, and, thus, determine the cytotoxicity thereof. For example, cell proliferation can be assayed by measuring Bromodeoxyuridine (BrdU) incorporation (see, *e.g.*, Hoshino et al., 1986, Int. J. Cancer 38, 369; Campana et al., 1988, J. Immunol. Meth. 107:79), (^{3}H) thymidine incorporation (see, *e.g.*, Chen, J., 1996, Oncogene 13:1395-403; Jeoung, J., 1995, J. Biol. Chem. 270:18367 73), by direct cell count, or by detecting changes in transcription, translation or activity of known genes such as protooncogenes (*e.g.*, fos, myc) or cell cycle markers (Rb, cdc2, cyclin A, D1, D2, D3, E, etc). The levels of such protein and mRNA and activity can be determined by any method well known in the art. For example, protein can be quantitated by known immunodiagnostic methods such as ELISA, Western blotting or immunoprecipitation using antibodies, including commercially available antibodies. mRNA can be quantitated using methods that are well known and routine in the art, for example, using northern analysis, RNase protection, or polymerase chain reaction in connection with reverse transcription. Cell viability can be assessed by using trypan-blue staining or other cell death or viability markers known in the art. In a specific embodiment, the level of cellular ATP is measured to determined cell viability.

[0307] In specific cases cell viability is measured in three-day and seven-day periods using an assay standard in the art, such as the CellTiter-Glo Assay Kit (Promega) which measures levels of intracellular ATP. A reduction in cellular ATP is indicative of a cytotoxic effect. In another specific case, cell viability can be measured in the neutral red uptake assay. In other cases, visual observation for morphological changes may include enlargement, granularity, cells with ragged edges, a filmy appearance, rounding, detachment from the surface of the well, or other changes. These changes

are given a designation of T (100% toxic), PVH (partially toxic-very heavy-80%), PH (partially toxic-heavy-60%), P (partially toxic-40%), Ps (partially toxic-slight-20%), or 0 (no toxicity-0%), conforming to the degree of cytotoxicity seen. A 50% cell inhibitory (cytotoxic) concentration (IC50) is determined by regression analysis of these data.

[0308] In a specific case, the cells used in the cytotoxicity assay are animal cells, including primary cells and cell lines. In some cases, the cells are human cells. In certain embodiments, cytotoxicity is assessed in one or more of the following cell lines: U937, a human monocyte cell line; primary peripheral blood mononuclear cells (PBMC); Huh7, a human hepatoblastoma cell line; 293T, a human embryonic kidney cell line; and THP-1, monocytic cells. In certain embodiments, cytotoxicity is assessed in one or more of the following cell lines: MDCK, MEF, Huh 7.5, Detroit, or human tracheobronchial epithelial (HTBE) cells.

[0309] A recombinant influenza virus provided herein, or a composition thereof can be tested for *in vivo* toxicity in animal models. For example, animal models known in the art can also be used to determine the *in vivo* toxicity of to test the activities of a recombinant influenza virus, an antibody or a composition thereof. For example, animals are administered a range of concentrations of to test the activities of a recombinant influenza virus, or a composition thereof. Subsequently, the animals are monitored over time for lethality, weight loss or failure to gain weight, and/or levels of serum markers that may be indicative of tissue damage (*e.g.*, creatine phosphokinase level as an indicator of general tissue damage, level of glutamic oxalic acid transaminase or pyruvic acid transaminase as indicators for possible liver damage). These *in vivo* assays may also be adapted to test the toxicity of various administration mode and/or regimen in addition to dosages.

[0310] The toxicity and/or efficacy of a recombinant influenza virus provided herein, or a composition thereof can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. A recombinant influenza virus, or a composition thereof that exhibits large therapeutic indices is preferred. While a recombinant influenza virus, or a composition thereof that exhibits toxic side effects may be used, care should be taken to design a delivery system that targets such agents to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[0311] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage of a recombinant influenza virus, or a composition thereof for use in humans. The dosage of such agents lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any active compound used in a method described herein, the effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high-performance liquid chromatography. Additional information concerning dosage determination is provided herein.

[0312] Further, any assays known to those skilled in the art can be used to evaluate the prophylactic and/or therapeutic utility of a recombinant influenza virus, an antibody or a composition thereof, for example, by measuring viral infection or a condition or symptoms associated therewith.

Animal Model Assays

[0313] The virulence of a recombinant influenza virus provided herein can be assessed in a subject, in particular an animal model. In one example, the ability to induce lung lesions and cause infection in an animal model of virus infection is compared to wild-type virus and mock virus. Lung lesions can be assessed as a percentage of lung lobes that are healthy by visual inspection. Animals are euthanized 5 days p.i. by intravenous administration of pentobarbital, and their lungs are removed in toto. The percentage of the surface of each pulmonary lobe that is affected by macroscopic lesions is estimated visually. The percentages are averaged to obtain a mean value for the 7 pulmonary lobes of each animal. In other assays, nasal swabs can be tested to determine virus burden or titer. Nasal swabs can be taken during necropsy to determine viral burden post-infection.

[0314] A recombinant influenza virus provided herein, or a composition thereof is preferably assayed *in vivo* for the desired therapeutic or prophylactic activity prior to use in humans. For example, to assess the use of a recombinant influenza virus, or a composition thereof to prevent an influenza virus disease, the virus, antibody or composition can be administered before the animal is infected with a wild-type influenza virus. Alternatively, or in addition, a recombinant influenza virus, or a composition thereof can be administered to the animal at the same time that the animal is infected with a wild-type influenza virus. To assess the use of a recombinant influenza virus, or a composition thereof to treat an influenza virus infection or disease associated therewith, the virus, or composition may be administered after infecting the animal with wild-type influenza virus. In a specific embodiment, a recombinant influenza virus, or a composition

thereof is administered to the animal more than one time.

[0315] A recombinant influenza virus provided herein, or a composition thereof can be tested for antiviral activity in animal model systems including, but are not limited to, rats, mice, chicken, cows, monkeys, pigs, goats, sheep, dogs, rabbits, guinea pigs, etc. In a specific embodiment, active compounds and compositions thereof are tested in a mouse model system. Such model systems are widely used and well-known to the skilled artisan. In a specific case, a recombinant influenza virus, or a composition thereof is tested in a mouse model system. Non-limiting examples of animal models for influenza virus are provided in this section.

[0316] In general, animals are infected with wild-type influenza virus and concurrently or subsequently treated with a recombinant influenza virus provided herein, or a composition thereof, or placebo. Alternatively, animals are treated with a recombinant influenza virus, or a composition thereof, or placebo and subsequently infected with wild-type influenza virus. Samples obtained from these animals (*e.g.*, serum, urine, sputum, semen, saliva, plasma, or tissue sample) can be tested for viral replication via well known methods in the art, *e.g.*, those that measure altered viral titers (as determined, *e.g.*, by plaque formation), the production of viral proteins (as determined, *e.g.*, by Western blot, ELISA, or flow cytometry analysis) or the production of viral nucleic acids (as determined, *e.g.*, by RT-PCR or northern blot analysis). For quantitation of virus in tissue samples, tissue samples are homogenized in phosphate-buffered saline (PBS), and dilutions of clarified homogenates are adsorbed for 1 hour at 37°C onto monolayers of cells (*e.g.*, Vero, CEF or MDCK cells). In other assays, histopathologic evaluations are performed after infection, preferably evaluations of the organ(s) the virus is known to target for infection. Virus immunohistochemistry can be performed using a viral-specific monoclonal antibody.

[0317] The effect of a recombinant influenza virus, provided herein or a composition thereof on the virulence of a virus can also be determined using *in vivo* assays in which the titer of the virus in an infected subject administered a recombinant influenza virus, or a composition thereof, the length of survival of an infected subject administered a recombinant influenza virus, or a composition thereof, the immune response in an infected subject administered a recombinant influenza virus, or a composition thereof, the number, duration and/or severity of the symptoms in an infected subject administered a recombinant influenza virus, or a composition thereof, and/or the time period before onset of one or more symptoms in an infected subject administered a recombinant influenza virus, or a composition thereof, is assessed. Techniques known to one of skill in the art can be used to measure such effects.

[0318] Influenza virus animal models, such as ferret, mouse, guinea pig, and chicken, developed for use to test antiviral agents against influenza virus have been described. See, e.g., Sidwell et al., Antiviral Res., 2000, 48:1-16; Lowen A.C. et al. PNAS., 2006, 103: 9988-92; and McCauley et al., Antiviral Res., 1995, 27:179-186. For mouse models of influenza, non-limiting examples of parameters that can be used to assay antiviral activity of active compounds administered to the influenza-infected mice include pneumonia-associated death, serum α1-acid glycoprotein increase, animal weight, lung virus assayed by hemagglutinin, lung virus assayed by plaque assays, and histopathological change in the lung. Statistical analysis is carried out to calculate significance (e.g., a P value of 0.05 or less).

[0319] In one example, the ability to induce lung lesions and cause infection in an animal model of virus infection is compared using wild-type virus and mock virus. Lung lesions can be assessed as a percentage of lung lobes that are healthy by visual inspection. Animals are euthanized 5 days p.i. by intravenous administration of pentobarbital, and their lungs are removed in toto. The percentage of the surface of each pulmonary lobe that is affected by macroscopic lesions is estimated visually. The percentages are averaged to obtain a mean value for the 7 pulmonary lobes of each animal. In other assays, nasal swabs can be tested to determine virus burden or titer. Nasal swabs can be taken during necropsy to determine viral burden post-infection.

[0320] In one case, virus is quantified in tissue samples. For example, tissue samples are homogenized in phosphate-buffered saline (PBS), and dilutions of clarified homogenates adsorbed for 1 h at 37°C onto monolayers of cells *(e.g.,* MDCK cells). Infected monolayers are then overlaid with a solution of minimal essential medium containing 0.1% bovine serum albumin (BSA), 0.01% DEAE-dextran, 0.1% NaHCO3, and 1% agar. Plates are incubated 2 to 3 days until plaques could be visualized. Tissue culture infectious dose (TCID) assays to titrate virus from PR8-infected samples are carried out as follows. Confluent monolayers of cells *(e.g.,* MDCK cells) in 96-well plates are incubated with log dilutions of clarified tissue homogenates in media. Two to three days after inoculation, 0.05-ml aliquots from each well are assessed for viral growth by hemagglutination assay (HA assay).

Assays in Humans

[0321] In one case, a recombinant influenza virus, provided herein or a composition thereof is assessed in infected human subjects. In this case, a recombinant influenza virus, or a composition thereof is administered to the human subject, and the effect of the virus, or composition on viral replication is determined by, *e.g.,* analyzing the level of the virus or viral nucleic acids in a biological sample (*e.g.,* serum or plasma). A recombinant influenza virus, or a composition thereof that alters virus replication can be identified by comparing the level of virus replication in a subject or group of subjects treated with a control to that in a subject or group of subjects treated with a recombinant influenza virus, or a composition thereof. Alternatively, alterations in viral replication can be identified by comparing the level of the virus

replication in a subject or group of subjects before and after the administration of a recombinant influenza virus, or a composition thereof. Techniques known to those of skill in the art can be used to obtain the biological sample and analyze the mRNA or protein expression.

[0322] In another case, the effect of a recombinant influenza virus, provided herein or a composition thereof on the severity of one or more symptoms associated with an influenza virus infection/disease are assessed in an infected subject. In this case, a recombinant influenza virus, or a composition thereof, or a control is administered to a human subject suffering from influenza virus infection and the effect of the virus, or composition on one or more symptoms of the virus infection is determined. A recombinant influenza virus, or a composition thereof that reduces one or more symptoms can be identified by comparing the subjects treated with a control to the subjects treated with the virus, or composition. Techniques known to physicians familiar with infectious diseases can be used to determine whether an ative compound or composition thereof reduces one or more symptoms associated with the influenza virus disease.

[0323] For quantitation of virus in tissue samples, tissue samples are homogenized in phosphate-buffered saline (PBS), and dilutions of clarified homogenates adsorbed for 1 h at 37°C onto monolayers of cells (*e.g.,* CEF or MDCK cells). Infected monolayers are then overlaid with a solution of minimal essential medium containing 0.1% bovine serum albumin (BSA), 0.01% DEAE-dextran, 0.1% $NaHCO_3$, and 1% agar. Plates are incubated 2 to 3 days until plaques could be visualized. Tissue culture infectious dose (TCID) assays to titrate virus from PR8-infected samples are carried out as follows. Confluent monolayers of cells (*e.g.,* CEF or MDCK cells) in 96-well plates are incubated with log dilutions of clarified tissue homogenates in media. Two to three days after inoculation, 0.05-ml aliquots from each well are assessed for viral growth by hemagglutination assay (HA assay).

[0324] In yet other assays, histopathologic evaluations are performed after infection. Nasal turbinates and trachea may be examined for epithelial changes and subepithelial inflammation. The lungs may be examined for bronchiolar epithelial changes and peribronchiolar inflammation in large, medium, and small or terminal bronchioles. The alveoli are also evaluated for inflammatory changes. The medium bronchioles are graded on a scale of 0 to 3+ as follows: 0 (normal: lined by medium to tall columnar epithelial cells with ciliated apical borders and basal pseudostratified nuclei; minimal inflammation); 1+ (epithelial layer columnar and even in outline with only slightly increased proliferation; cilia still visible on many cells); 2+ (prominent changes in the epithelial layer ranging from attenuation to marked proliferation; cells disorganized and layer outline irregular at the luminal border); 3+ (epithelial layer markedly disrupted and disorganized with necrotic cells visible in the lumen; some bronchioles attenuated and others in marked reactive proliferation).

[0325] The trachea is graded on a scale of 0 to 2.5+ as follows: 0 (normal: Lined by medium to tall columnar epithelial cells with ciliated apical border, nuclei basal and pseudostratified. Cytoplasm evident between apical border and nucleus. Occasional small focus with squamous cells); 1+ (focal squamous metaplasia of the epithelial layer); 2+ (diffuse squamous metaplasia of much of the epithelial layer, cilia may be evident focally); 2.5+ (diffuse squamous metaplasia with very few cilia evident).

[0326] Virus immunohistochemistry is performed using a viral-specific monoclonal antibody (*e.g.* NP-, N- or HN-specific monoclonal antibodies). Staining is graded 0 to 3+ as follows: 0 (no infected cells); 0.5+ (few infected cells); 1+ (few infected cells, as widely separated individual cells); 1.5+ (few infected cells, as widely separated singles and in small clusters); 2+ (moderate numbers of infected cells, usually affecting clusters of adjacent cells in portions of the epithelial layer lining bronchioles, or in small sublobular foci in alveoli); 3+ (numerous infected cells, affecting most of the epithelial layer in bronchioles, or widespread in large sublobular foci in alveoli).

5.9 SCREENING ASSAYS

[0327] A recombinant influenza virus described herein may be used t o study the life cycle of an influenza virus. For example, a recombinant influenza virus described herein that expresses a detectable heterologous sequence (e.g., a detectable substance such as GFP or luciferase, or another detectable substance described herein or known in the art) is introduced into a host cell and the life cycle of the virus is monitored by the assessing the expression of the detectable heterologous sequence. A recombinant influenza virus described herein that expresses a detectable heterologous sequence may also be administered to a non-human animal and the infection monitored by assessing the expression of the detectable heterologous sequence. In certain cases, the recombinant influenza virus is a nine segmented influenza virus described herein.

[0328] Described herein are high throughput screening assays for the identification or validation of compounds that modulate the replication of influenza viruses. In a specific case, the high throughput screening assay to identify a compound that modulates the replication of an influenza virus comprises: (a) contacting a compound or a member of a library of compounds with a host cell infected with a recombinant influenza virus described herein that expresses a detectable heterologous nucleotide sequence; and (b) measuring the expression or activity of a product encoded by the detectable heterologous nucleotide sequence. In another case, the high throughput screening assay to identify a compound that modulates the replication of an influenza virus comprises: (a) infecting a host cell with a recombinant influenza virus described herein that expresses a detectable heterologous nucleotide sequence in the presence of a compound

or a member of a library of compounds; and (b) measuring the expression or activity a product encoded by the detectable heterologous nucleotide sequence. In another case, the high throughput screening assay to identify a compound that modulates the replication of an influenza virus comprises: (a) contacting a host cell with a compound or a member of a library of compounds; (b) infecting the host cell with a recombinant influenza virus described herein that expresses a detectable heterologous nucleotide sequence; and (c) measuring the expression or activity a product encoded by the detectable heterologous nucleotide sequence. In a specific case, the recombinant influenza virus is a nine-segmented influenza virus described herein.

[0329] In some cases, the high throughput screening assays involve: (a) contacting a compound or a member of a library of compounds with a cell before (*e.g.,* 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours or more before), concurrently and/or subsequent to (*e.g.,* 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours, 12 hours, 24 hours or more after) infection with a recombinant influenza virus described herein that expresses a detectable heterologous nucleotide sequence; and (b) measuring the expression or activity a product encoded by the detectable heterologous nucleotide sequence. The cells can be infected with different MOIs (*e.g.,* 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 2.5, or 5) and the effect of compounds can be assessed in the screening assays. The effect of different concentrations of the compounds can also be assessed using in the screening assays. The expression or activity of a product encoded by the detectable heterologous nucleotide sequence can be measured at different times post-infection. For example, the expression or activity of the detectable heterologous nucleotide sequence may be measured 6 hours, 12 hours, 24 hours, 48 hours or 72 hours post-infection. A compound that increases the replication of an influenza virus is identified if the level of expression or activity a product encoded by the detectable heterologous nucleotide sequence is increased in the host cell contacted with the compound relative to the level of expression or activity a product encoded by the detectable heterologous nucleotide sequence in a host cell contacted with a negative control (e.g., an influenza virus that is not a recombinant influenza virus described herein (e.g., a wild-type influenza virus)). In contrast, a compound that decreases the replication of an influenza virus is identified if the level of expression or activity a product encoded by the detectable heterologous nucleotide sequence is decreased in the host cell contacted with the compound relative to the level of expression or activity of a product encoded by the detectable heterologous nucleotide sequence in a host cell contacted with a negative control (e.g., an influenza virus that is not a recombinant influenza virus described herein (e.g., a wild-type influenza virus)). In some cases, an embryonated egg or any other substrate that permits the replication of an influenza virus may be used in place of the cells used in the high throughput screening assays described herein.

[0330] In a specific case, the host cell used in the high throughput assay for screening for compounds that modulate replication of an influenza virus is a host cell that permits infection with the influenza virus. In some cases, the high throughput assay for screening for compounds that modulate replication of an influenza virus uses any substrate that allows the virus to grow to titers that permits the use of such viruses in the screening assays. By way of non-limiting example, substrates useful in the high throughput screening assays described herein include cells (*e.g.* avian cells, chicken cells *(e.g.,* primary chick embryo cells or chick kidney cells), Vero cells, MDCK cells, human respiratory epithelial cells (*e.g.,* A549 cells), calf kidney cells, mink lung cells, etc.) that are susceptible to infection by the viruses or embryonated eggs (e.g., embryonated chick eggs 6 to 9 days old, 6 to 10 days old, 10 to 12 days, or 10 to 14 days old) or animals (e.g., birds). In one embodiment, the cells used in the high throughput screening assay are biologically relevant to the type of infection.

[0331] In a specific case, a product encoded by the detectable heterologous nucleotide sequence measured in the high throughput screening assays described above is an RNA product. In another case, a product encoded by the detectable heterologous nucleotide sequence measured in the high throughput screening assays described above is a protein product. In another case, the activity of a product encoded by the detectable heterologous nucleotide sequence is measured in the high throughput screening assays described above and the product is protein.

[0332] Any method known to one of skill in the art can be used measure the expression or activity of a product encoded by the detectable heterologous nucleotide sequence. In one case, the product encoded by the detectable heterologous nucleotide sequence is RNA and a technique known to one of skill in the art, such as RT-PCR or Northern blot analysis, is used to measure the expression of the RNA product. In another case, the product encoded by the detectable heterologous nucleotide sequence is protein and a technique known to one of skill in the art, such as western blot analysis or an ELISA, is used to measure the expression of the protein product. In another case, the product encoded by the detectable heterologous nucleotide sequence is protein and the activity of the protein is measured using a technique known to one of skill in the art.

[0333] Any screening assay described herein can be performed individually, e.g., just with the test compound, or with appropriate controls. For example, a parallel assay without the test compound, or other parallel assays without other reaction components (e.g., virus) can be performed. In one case, a parallel screening assay as described above is performed except that a negative control and/or a positive control are used in place of a test compound. In another case, to eliminate cytotoxic compounds that appear as false positives, a counter screen is performed in which uninfected cells are transfected with a nucleic acid construct (*e.g.,* a plasmid) comprising a detectable heterologous nucleotide sequence and the expression or activity of a product encoded by the detectable heterologous nucleotide sequence is measured.

Alternatively, it is possible to compare assay results to a reference, *e.g.,* a reference value, *e.g.,* obtained from the literature, a prior assay, and so forth. Appropriate correlations and art known statistical methods can be used to evaluate an assay result.

[0334] In some cases, the average expression or activity of the product encoded by the detectable heterologous nucleotide sequence when a negative control (e.g., PBS) is contacted with cell is determined and the percent expression or activity of the product for each compound is determined in relation to this internal control. In one case, the average percent expression or activity the product encoded by the detectable heterologous nucleotide sequence is calculated and the compounds are classified as strong or medium inhibitors of virus replication based on a 90% to 100% or 70% to 89% reduction in the expression or activity of the product, respectively. In another case, the compounds are classified as enhancers of viral replication if at least a 2 fold increase in the expression or activity of a product encoded by the detectable heterologous nucleotide sequence above relative to the negative control is obtained.

[0335] The antiviral effect of a compound on influenza virus can be assessed in a non-human animal using a recombinant influenza virus described herein. In one case, the antiviral effect of a compound on influenza virus can be assessed by a method comprising: (a) administering (for example, parenterally, subcutaneously, intranasally, or intraperitoneally) to a non-human subject, concurrently, subsequently or prior to administration of a compound, an effective amount of a recombinant influenza virus described herein; b) waiting for a time interval following the administration of the recombinant influenza virus; and d) detecting the recombinant influenza virus in the subject or in a biological specimen from the subject. In a specific case, the recombinant influenza virus is a nine-segmented influenza virus described herein.

5.10 KITS

[0336] In one aspect, provided herein is a kit comprising, in one or more containers, one or more nucleic acid sequences described herein. In a specific embodiment, a kit comprises, in one, two or more containers, one, two or more chimeric influenza virus gene segments or the complements thereof. In another case, a kit comprises, in one, two or more containers, one or more nucleic acid sequences encoding one, two or more chimeric influenza virus gene segments or the complements thereof. The kit may further comprise one or more of the following: host cells suitable for rescue of the virus, reagents suitable for transfecting plasmid DNA into a host cell, helper virus, plasmids encoding one or more types of influenza virus gene segments, one or more expression plasmids encoding viral proteins, and/or one or more primers specific for one, two or more chimeric influenza virus gene segments or the complements thereof, or nucleic acid sequences encoding the same.

[0337] In certain cases, a kit comprises, in one, two or more containers, nucleic acid sequences comprising or encoding a combination of: (i) the following or the complement thereof from one type of influenza virus gene segment: 5' and 3' non-coding regions and either a 3' proximal coding region sequence with any start codon eliminated so that it is not translated, a 5' proximal coding region sequence that is not translated, or both a 3' proximal coding region sequence with any start codon eliminated so that it is not translated and a 5' proximal coding region sequence that is not translated; and (ii) either at least the 3' proximal 20 nucleotides of an open reading frame from a different type of influenza virus gene segment or the complement thereof with one, two three or more mutations, at least the 5' proximal 30 nucleotides of an open reading frame from a different type of influenza virus gene segment or the complement thereof with one, two, three or more mutations, or both the at least 3' proximal 20 nucleotides of an open reading frame and at least the 5' proximal 30 nucleotides of an open reading frame from a different type of influenza virus gene segment or the complement thereof with one, two, three or more mutations. In some cases, such nucleic acid sequences may be used as a template to engineer in a nucleotide sequence (*e.g.,* a heterologous nucleotide sequence) which is in frame with the 3' proximal 20 nucleotides and/or the 5' proximal 30 nucleotides of the open reading frame from the different type of influenza virus gene segment. The chimeric influenza virus gene segment or complement thereof, or a nucleic acid encoding the gene segment or complement thereof may contain one, two or more restriction enzyme sites that would enable the incorporation of a nucleotide sequence (*e.g.,* a heterologous nucleotide sequence) in frame with the 3' and/or 5' proximal nucleotides of the open reading frame of the different type of influenza virus gene segment. In certain embodiments, such kits further comprise one or more restriction enzymes that cleave the nucleic acid sequence.

[0338] Described herein is a kit comprising one or more containers filled with one or more of the one or more recombinant influenza virus described herein or a composition thereof. Described herein is a pharmaceutical pack or kit comprising, in one or more containers, a composition comprising one or more recombinant influenza viruses described herein. Described herein is a kit comprising, in one or more containers, primers specific for a particular chimeric influenza virus gene segment.

[0339] Described herein is a kit comprising one or more containers filled with one or more antibodies generated or identified using a recombinant influenza virus described herein. In one case, a kit comprises an antibody described herein, preferably an isolated antibody, in one or more containers. In a specific case, a kit encompassed herein contains an isolated influenza virus antigen that the antibodies encompassed herein react with as a control. In a specific, a kit provided herein further comprise a control antibody which does not react with an influenza virus antigen that an antibody

encompassed herein reacts with. In another specific case, a kit provided herein contains a means for detecting the binding of an antibody to an influenza virus antigen that an antibody encompassed herein reacts with (*e.g.,* the antibody may be conjugated to a detectable substrate such as a fluorescent compound, an enzymatic substrate, a radioactive compound or a luminescent compound, or a second antibody which recognizes the first antibody may be conjugated to a detectable substrate). In specific cases, a kit may include a recombinantly produced or chemically synthesized influenza virus antigen. The influenza virus antigen provided in the kit may also be attached to a solid support. In a more specific embodiment the detecting means of the above described kit includes a solid support to which an influenza virus antigen is attached. Such a kit may also include a non-attached reporter-labeled anti-human antibody. In this embodiment, binding of the antibody to the Influenza virus antigen can be detected by binding of the said reporter-labeled antibody.

[0340] Optionally associated with such a kit can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

6. EXAMPLE 1

[0341] This example describes the production of chimeric influenza virus gene segments and the use of those gene segments to produce an influenza virus that is not able to reassort with other influenza viruses to produce replicating reassortant virus.

6.1 MATERIALS & METHODS

[0342] **Cells and viruses.** 293T cells were maintained in Dulbecco's modified Eagle's medium with 10 % fetal calf serum. MDCK cells were grown in Eagle's minimal essential medium with 10 % fetal calf serum. Viruses were grown in 10-day-old specific-pathogen-free chicken embryos (Charles River Laboratories, SPAFAS, Preston, CT).

[0343] **Plasmid construction.** (i) Generation of NS-HAwt-NS construct (Figure 25A). The 1.2 kb Kpn I fragment from the previously constructed pDZ-NS plasmid (Quinlivan M, et al. (2005) Attenuation of equine influenza viruses through truncations of the NS1 protein. J Virol 79:8431-8439) was transferred to the Kpn I site of pUC18 vector and subjected to site-directed mutagenesis to mutate two ATGs (A27T, A76T), one splice site (G57C), and to generate one Nhe I site (A104G, G109C) and one Xho I site (G759C, A760G). The 1.2 kb NS Kpn I fragment was then transferred back to the pDZ vector (Quinlivan M, et al. (2005) Attenuation of equine influenza viruses through truncations of the NS1 protein. J Virol 79:8431-8439) (in which the Nhe I and Xho I sites have been removed), resulting in a plasmid pDZ-NS-ps. The ORF of the A/PR/8/34 HA protein, which is 1,698 bp long, was amplified from an ambisense pDZ-HA plasmid (Quinlivan M, et al. (2005) Attenuation of equine influenza viruses through truncations of the NS1 protein. J Virol 79:8431-8439) and subjected to mutagenesis to mutate an internal Xho I site (C143G). Two restriction sites, Nhe I and Xho I, were introduced to flank the HA ORF, which was then used to replace the Nhe I and Xho I fragment of the NS ORF of pDZ-NS-ps plasmid to form the NS-HAwt-NS construct (Figure 25A). (ii) Generation of HA-NSwt-HA construct (Figure 25A). Using the same strategy, three ATGs were mutated on the 3' HA packaging signal (A33T, A79T and A92T). The ORF of the A/PR/8/34 NS proteins (NS1, NS2), which is 838 bp long, was amplified and ligated to the HA packaging sequences in a pDZ vector to form the HA-NSwt-HA construct (Figure 25A). (iii) Generation of NS-HAmut-NS construct (Figure 26A). The method was the same as described for NS-HAwt-NS (Figure 25A) except that the primers used to amplify the HA ORF carried synonymous mutations. The forward primer is: 5'-ca gctagc atg aaA gcG aaT TtG TtA gtT TtA CtG TCC gcG TtG gcG gcC gcG gaC gca gac aca ata tgt ata ggc tac c-3' (SEQ ID NO:114); and the two reverse primers are 5'-cca Aaa GGA Aat Cgc Tcc TaA ACT Aac TaG CaA Tac TaA GCT GGA Age gac agt tga gta gat cgc c-3'(SEQ ID NO:115) and 5'-gt ctcgag tca Aat Aca Aat CcG Aca Ttg TaG GCT Ccc Gtt GCT Gca cat cca Aaa GGA Aat Cgc Tcc TaA AC-3' (SEQ ID NO:116). (iv) Generation of the HA-NSmut-HA construct (Figure 26A). The method was also the same as described for HA-NSwt-HA (Figure 25A) except that synonymous mutations were introduced into the NS ORF. The forward primer is: 5'-ca gctagc atg gaC ccG aaT acC gtA AGT TCT ttt cag gta gaC tgc ttt ctt tgg cat gtc c-3' (SEQ ID NO:117); the reverse primer is: 5'-gt ctcgag tta Gat CaA Ttg Gaa GCT Aaa Ggt CcG Gat Ttc Ctg etc cac ttc aag c-3' (SEQ ID NO:118). (The capitalized letters in these primer sequences designate mutated nucleotides.)

[0344] **Reverse genetics for recombinant viruses.** The method for generating recombinant influenza viruses was slightly modified from previous protocols (Gao Q, Brydon EW, Palese P (2008) A seven-segmented influenza A virus expressing the influenza C virus glycoprotein HEF. J Virol 82:6419-6426, Quinlivan M, et al. (2005) Attenuation of equine influenza viruses through truncations of the NS1 protein. J Virol 79:8431-8439; Fodor E, et al. (1999) Rescue of influenza A virus from recombinant DNA. J Virol 73:9679-9682). For the generation of the Swap(wt) and Swap(mut) viruses (Figures 25B & 26B), 293T cells were transfected with six A/PR/8/34 plasmids (pDZ-PB2, PB1, PA, NP, NA, M), and the two chimeric HA and NS constructs [NS-HAwt-NS and HA-NSwt-HA, or NS-HAmut-NS and HA-NSmut-HA] (Figures 25A & 26A). For the generation of the Reassortant(NS) virus (Figure 25C), 293T cells were transfected with seven A/PR/8/34 plasmids (pDZ-PB2, PB1, PA, HA, NP, NA, M), and the HA-NSwt-HA construct. Seven A/PR/8/34 plasmids

(pDZ-PB2, PB1, PA, NP, NA, M, NS), and the NS-HAwt-NS construct were used to rescue the Reassortant(HA) virus (Figure 25D).

[0345] **Acrylamide gel electrophoresis of purified vRNA.** The viruses were grown in 10-day-old eggs at 37°C and were subsequently processed by using a previously reported method (Gao Q, Brydon EW, Palese P (2008) A seven-segmented influenza A virus expressing the influenza C virus glycoprotein HEF. J Virol 82:6419-6426). Briefly, virus was purified and RNA was isolated and run on a 2.8 % denaturing polyacrylamide gel which was then stained with a silver staining kit (Invitrogen).

[0346] **Immunostaining of plaques.** Previous methods were followed (Gao Q, Brydon EW, Palese P (2008) A seven-segmented influenza A virus expressing the influenza C virus glycoprotein HEF. J Virol 82:6419-6426; Matrosovich M, Matrosovich T, Garten W, Klenk HD (2006) New low-viscosity overlay medium for viral plaque assays. Virol J 3:63). A rabbit anti-A/PR/8/34 polyclonal antibody (1:2,000 dilution) was used for plaque visualization.

[0347] **Viral growth kinetics.** 10-day-old embryonated chicken eggs were inoculated with influenza viruses (100 PFU/egg) and incubated at 37°C. At 24, 48 and 72 hr post inoculation, the allantoic fluids were harvested and the titers of the viruses were determined by plaque assay or immunostaining of the plaques in MDCK cells. At each time point, three eggs were analyzed for each virus.

6.2 RESULTS

[0348] A chimeric influenza A virus segment containing the ORF of the HA gene and the packaging signals from the NS gene, and a chimeric influenza A virus segment containing the ORF of the NS gene and the packaging sequences of the HA gene were generated and used to construct a recombinant influenza virus. To do this, the wild type HA ORF was amplified by polymerase chain reaction (PCR), and ligated to the flanking NS packaging sequences which include: the 3' and 5' NCRs, the 3' seventy seven nt, and the 5' one hundred and two nt of the NS ORF. This generated the chimeric NS-HAwt-NS construct of 1941 nt in length (Figure 25A). The two translation initiation codons and one splice site in the 77 nt of the NS 3' ORF packaging signal were mutated in order to allow the HA to translate from its own start codon (Figure 25A). Following the same strategy, a 1099 nt long HA-NSwt-HA construct was also made (Figure 25A). In this construct, the NS ORF - which encodes both NS1 and NS2 proteins - was flanked by the 3' and 5' NCRs of the HA, the 3' sixty seven nt, and the 5' one hundred and five nt of the HA ORF. The three start codons located in the 67 nt of the 3' ORF packaging region of the HA were also mutated. Since the A/PR/8/34 virus was used as a backbone and since the currently known HA and NS packaging signals were all identified in the A/WSN/33 virus (Fujii K, et al. (2005) Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions. J Virol 79:3766-3774; Watanabe T, Watanabe S, Noda T, Fujii Y, Kawaoka Y (2003) Exploitation of nucleic acid packaging signals to generate a novel influenza virus-based vector stably expressing two foreign genes. J Virol 77:10575-10583), the flanking packaging sequences used in these experiments were made slightly longer than those identified in A/WSN/33 in order to assure proper packaging.

[0349] Using previously established methods, the Swap(wt) virus was successfully rescued and was shown to be stable for multiple passages in embryonated chicken eggs (Figure 25B). This virus contains six A/PR/8/34 wild type segments (PB2, PB1, PA, NP, NA, and M) and the two chimeric segments: NS-HAwt-NS and HA-NSwt-HA (Figure 25B). The Swap(wt) virus grew well in eggs, and titers could reach more than 10^8 plaque forming units per ml (PFU/ml) one day post inoculation (Figure 25F). Nevertheless, it was still slightly attenuated in growth compared to the recombinant A/PR/8/34 virus. In Madin-Darby canine kidney (MDCK) cells, the plaques formed by the Swap(wt) virus were slightly smaller than those of A/PR/8/34 virus (Figure 25E), while in eggs, the titers of the Swap(wt) virus were about 10-fold lower than those of A/PR/8/34 virus (Figure 25F).

[0350] In order to determine whether the HA-NSwt-HA and NS-HAwt-NS segments could each freely reassort with wild type virus genes, viruses were constructed which carried just one of these chimeric genes. Surprisingly, two recombinant viruses were rescued: Reassortant(NS) and Reassortant(HA) (Figures 25C & 25D). The Reassortant(NS) virus contains seven A/PR/8/34 segments (PB2, PB1, PA, HA, NP, NA, and M) and one chimeric HA-NSwt-HA segment (Figure 25C); the Reassortant(HA) virus has seven A/PR/8/34 vRNAs (PB2, PB1, PA, NP, NA, M and NS) and one chimeric NS-HAwt-NS segment (Figure 25D). Interestingly, the Reassortant(NS) virus exhibited efficient growth (Figure 25F). The plaque sizes in MDCK cells and the titers in eggs were both similar to those of the Swap(wt) virus (Figures 25E & 25F). The Reassortant(HA) virus was more attenuated, with smaller plaques in MDCK cells and lower titers in eggs (Figures 25E & 25F). The rescue of both viruses indicated that each of the chimeric segments of the Swap(wt) virus could independently reassort to form a reassortant virus.

[0351] The ability of the NS-HAwt-NS or HA-NSwt-HA segment to independently form a reassortant virus could be due to the fact that two sets of segment-specific packaging signals co-exist on the same vRNA (Figure 25). The NS-HAwt-NS segment still maintains its original HA-specific packaging sequences in its HA ORF region in addition to the flanking NS packaging signals (Figure 25A). The same is true for the HA-NSwt-HA segment. The original packaging signals may still be functional (Figure 25A). Considering this possibility, serial synonymous mutations were introduced

into the 3' and 5' ends of the ORFs in these chimeric constructs in order to force utilization of the flanking packaging signals only (Figure 26A). Previous studies have showed that the serial synonymous mutations in the coding region packaging sequences of the HA and NS segments indeed diminished the vRNA packaging efficiency (Fujii K, et al. (2005) Importance of both the coding and the segment-specific noncoding regions of the influenza A virus NS segment for its efficient incorporation into virions. J Virol 79:3766-3774; Marsh GA, Hatami R, Palese P (2007) Specific residues of the influenza A virus hemagglutinin viral RNA are important for efficient packaging into budding virions. J Virol 81:9727-9736). In this study, 22 and 45 nt mutations were introduced to the 3' and 5' ends of the HA ORF, respectively, forming a new construct NS-HAmut-NS (Figure 26A, and materials and methods); a similar method was applied to the HA-NSwt-HA and 12 and 15 nt mutations were introduced to construct the HA-NSmut-HA (Figure 26A, and materials and methods).

[0352] By using the same procedure as that in Figure 25B, the Swap(mut) virus (Figure 26B), which contains six A/PR/8/34 segments (PB2, PB1, PA, NP, NA, and M) and the two chimeric segments NS-HAmut-NS and HA-NSmut-HA (Figure 26B), was successfully rescued. Right after the rescue, the titer of the Swap(mut) virus was low. After one passage in eggs, the virus grew to higher titers and maintained the same yield over multiple passages. The plaque sizes of the Swap(mut) virus were similar to those of the Swap(wt) virus (Figures 25E & 26C). However, in eggs, the Swap(mut) virus grew slightly better than Swap(wt), although it was still slightly attenuated compared to the A/PR/8/34 virus (Figure 26D). It should be noted that eight and two nucleotide conversions were identified on the 3' ends of the NS-HAmut-NS and HA-NSmut-HA vRNAs of the passaged virus, respectively (see Figure 26B legend).

[0353] In order to determine whether the chimeric genes in Figure 26A are able to independently reassort with wild type ones, the rescue of two viruses was attempted (shown in Figure 26E). The genetic compositions of these two viruses are similar to those of the Reassortant(NS) (Figure 25C) and the Reassortant(HA) (Figure 25D) viruses, except that now the HA-NSmut-HA and NS-HAmut-NS constructs (Figure 26A) have been substituted for their counterparts (see Figure 26E). If each chimeric segment still maintains its ability to reassort freely, then the two viruses in Figure 26E should have been rescued. However, while the Reassortant(NS) (Figure 25C) and Reassortant(HA) viruses (Figure 1D) were easily rescued, neither of the viruses shown in Figure 26E could be obtained. The failure of the rescue suggests that, unlike HA-NSwt-HA and NS-HAwt-NS, the HA-NSmut-HA and NS-HAmut-NS segments cannot freely reassort with wild type genes.

[0354] Five recombinant viruses [rA/PR/8/34 (Figure 27A), Swap(wt) (Figure 27B) , Reassortant(NS) (Figure 27C), Reassortant(HA) (Figure 27D) and Swap(mut) (Figure 27E)] were grown in eggs and concentrated through a 30% sucrose cushion. RNA was isolated from purified virus and resolved on a 2.8% acrylamide gel to visualize the virus genome composition by silver staining. The NS-HAwt-NS segment of the Swap(wt) virus was inefficiently packaged while the other chimeric segment HA-NSwt-HA has better packaging efficiency (Figure 27B). For the two reasssortant viruses [Reassortant(NS) and Reassortant(HA)], neither chimeric segment [HA-NSwt-HA in Figure 27C and NS-HAwt-NS in Figure 27D] was efficiently incorporated. The packaging efficiency of the NS-HAwt-NS segment of the Reassortant(HA) virus was very low (Figure 27D), which might explain the attenuation observed in both MDCK cells and eggs (Figures 25E & 25F). The two chimeric segments of the Swap(mut) virus were efficiently incorporated compared to the other segments (Figure 27E). The NS-HAmut-NS segment of the Swap(mut) virus (Figure 27E) was incorporated more efficiently than the NS-HAwt-NS segment of the Swap(wt) virus (Figure 27B), suggesting that disruption of the original packaging signals of the HA ORF of the chimeric HA segment is critical to achieve efficient packaging. There was no significant difference in the levels of incorporation between HA-NSwt-HA and HA-NSmut-HA segments and both were packaged efficiently (Figures 27B & 27E).

[0355] Although the rescue of the two viruses in Figures 25C and 25D, but not the two hypothetical viruses in Figure 26E, did indicate which chimeric segment can freely form reassortant virus with wild type segments, these experiments per se did not directly assay reassortment. In order to determine whether the chimeric segments can freely reassort in tissue culture, MDCK cells were co-infected with the Swap(wt) [or Swap(mut)] virus and rA/PR/8/34 virus at an moi of 10 for each one (Figure 10A). Single plaques were isolated and subsequently amplified in MDCK cells. RNA was purified from amplified virus and RT-PCR was done to detect the HA and NS segments (Figure 28A). An 824 base pair (bp) product was observed for both the NS-HAwt-NS and NS-HAmut-NS segments, while for the rA/PR/8/34 HA, a 747 bp band was obtained (Figures 28B, 28D & 28E). The PCR products for chimeric and wild type NS segments, on the other hand, were 405 and 326 bp long, respectively (Figures 28C, 28D & 28E). For the Swap(wt) and rA/PR/8/34 co-infection experiment, 24 plaques were characterized, and two of them (plaques 3 and 8, indicated by arrows) showed reassortment of the HA-NSwt-HA segment with wild type virus (Figure 28D). The genetic makeup of these two plaques is the same as the Reassortant(NS) virus (Figure 25C). Reassortment of the NS-HAwt-NS segment was not observed, possibly due to its lower packaging efficiency (Figure 27D). For the Swap(mut) and rA/PR/8/34 co-infection experiment, 48 plaques were picked and they all contained wild type HA and NS genes, indicating the inability of NS-HAmut-NS or HA-NSmut-HA to reassort freely.

6.3 DISCUSSION

[0356] Interestingly, for the two chimeric constructs [NS-HAwt-NS and HA-NSwt-HA (Figure 25A)], each contained two sets of segment specific packaging sequences: the NS-HAwt-NS contained the NS-specific NCRs and ORF packaging regions in addition to the ORF packaging regions of the HA gene; the HA-NSwt-HA contained the HA-specific NCRs and ORF packaging regions in addition to the ORF packaging regions of the NS gene (Figure 25A). The efficient growth of the Swap(wt) virus in both MDCK cells and eggs indicates that two sets of signals can co-exist on one vRNA (Figures 25E & 25F). It is unclear, however, which set plays the major role during the genome recruitment process.

[0357] The levels of the NS-HAwt-NS RNA in the Swap(wt) (Figure 27B) and Reassortant(HA) (Figure 27D) viruses were significantly lower than those of the other segments. This suggests that two sets of signals may interfere with each other during the influenza RNA packaging process if they co-exist on one segment. This also suggests the incompatibility of two sets of packaging signals on one segment. The successful rescue of the two reassortant viruses [Reassortant (NS) (Figure 25C) & Reassortant (HA) (Figure 25D)] demonstrates that one virus can incorporate the same packaging signals twice. For example, the Reassortant(NS) virus contains two copies of HA packaging sequences derived from both the wild type HA segment and the HA-NSwt-HA chimeric segment (Figure 25C); the Reassortant(HA) virus carries two copies of NS packaging signals derived from both the wild type NS segment and the NS-HAwt-NS chimeric segment (Figure 25D). This phenomenon agrees with a previous finding that a nine-segmented influenza virus can incorporate two NS segments (Enami M, Sharma G, Benham C, Palese P (1991) An influenza virus containing nine different RNA segments, Virology 185:291-298).

[0358] The data presented herein show that, by simply flanking the ORF with packaging sequences from another segment, inhibition of reassortment cannot be achieved. It was possible to rescue viruses containing a single chimeric gene [the HA-NSwt-HA in the Reassortant(NS) virus (Figure 25C), and the NS-HAwt-NS in the Reassortant(HA) virus (Figure 25D)], and to identify in a reassortment experiment, viruses with a chimeric HA-NSwt-HA segment (Figure 28D). In the reassortment experiment, viruses with the NS-HAwt-NS segment or the Swap(wt) genotype were not isolated. This can be explained by the relatively low number of plaques analyzed. Considering the possibility that the ORF terminal packaging signals in the chimeric segments might still be functional, serial silent mutations were introduced into these signals and subsequently, each segment [NS-HAmut-NS or HA-NSmut-HA] lost its ability to freely reassort (Figures 26 & 28). Without being bound by any theory, the remaining flanking regions of these two chimeric segments become the main signals for packaging and as a result, the HA is recognized as an NS gene and the NS is recognized as an HA gene. Single reassortants with the NS-HAmut-NS or HA-NSmut-HA segment could not be rescued because such viruses would lack an HA or NS packaging signal. Also, in the tissue culture reassortment experiment, no single reassortant was isolated. However, a limitation of the experimental setup holds true for the reassortment between the Swap(mut) and rA/PR/8/34 viruses. Only 48 plaques were isolated and thus one cannot exclude the possibility that a virus with a single rewired segment could be formed. Nevertheless, the data suggest that rewiring of the packaging signals results in a deficiency for reassortment. Only viruses which contain a full complement of all eight packaging signals will grow to high yields. In the case of rewiring one segment by eliminating the original packaging signal, a virus will lose viability which can be regained only by rewiring a second segment to provide the missing packaging sequences. Thus, a virus with an HA gene with the NS packaging identity must also have an NS gene with the HA packaging identity.

[0359] Thus, this study offers a method for rewiring the influenza virus RNAs to prevent reassortment, which can be used for future live influenza vaccine constructions.

7. EXAMPLE 2

[0360] This example describes the production of recombinant influenza viruses using reverse genetics.

[0361] Three recombinant A/PR/8/34 viruses with 6 or 7 rewired RNA segments were successfully generated (Figs. 34-36). Each of the chimeric segments that carried packaging signals from a different segment either lost or significantly decreased its ability to form reassortant virus with wild type RNAs.

[0362] To generate the chimeric constructs used to rescue the recombinant viruses shown in Figs. 34-36, two sets of plasmids were used: one set of 8 plasmids carried the segment-specific packaging sequences derived from the 8 RNA segments of the influenza A/PR/8/34 virus (see Figs. 1-8). Importantly, the ATGs located on each 3' end-proximal ORF region packaging signal and the 5' splice site on the M and NS segment-derived packaging sequences were all mutated to allow for correct initiation of downstream ORFs (see Figs. 1-8); the second set of 8 plasmids carried all 8 ORFs of the influenza A/PR/8/34 virus segments. For each ORF, serial silent mutations were introduced to both the 3' and 5' ends of the ORFs to inactivate the ORF region packaging signals (see Figs. 9-16). All the 8 ORFs that carried silent mutations at the two ends were flanked by one Nhe I and one Xho I for ligation to the constructs carrying segment-specific packaging sequences. In addition, the pre-existing Nhe I or Xho I sites located on some ORF regions were all mutated by site-directed mutagenesis.

[0363] The method for generating recombinant influenza viruses was modified from that described in Example 1 and

in Gao and Palese, 2009, PNAS 106:15891. For the generation of the recombinant virus in Fig. 35, 293T cells were transfected with 6 chimeric plasmids (NA-PB2mut-NA, PB2-PB1mut-PB2, PB1-PAmut-PB1, M-NPmut-M, PA-NAmut-PA, NP-Mmut-NP), and 2 plasmids carrying the wild type A/PR/8/34 HA and NS segments. 24 hours post transfection, the cells were harvested and inoculated into 10-day-old specific-pathogen-free chicken embryos (Charles River Laboratories, SPAFAS, Preston, CT). Three days later, the allantoic fluids were harvested and an HA assay was used to determine the existence of rescued virus. The other two chimeric viruses shown in Figs. 36 and 37 were generated by using the same method. The virus in Fig. 36 contained 6 chimeric segments (NA-PB2mut-NA, PB2-PB1mut-PB2, PB1-PAmut-PB1, NS-HAmut-NS, PA-NAmut-PA, HA-NSmut-HA), and 2 wild type A/PR/8/34 NP and M segments. The virus in Fig. 37 contained 7 chimeric segments (NA-PB2mut-NA, PB2-PB1mut-PB2, PB1-PAmut-PB1, NP-HAmut-NP, NS-NPmut-NS, PA-NAmut-PA, HA-NSmut-HA), and 1 wild type A/PR/8/34 M segments. All these three chimeric viruses grew well, with titers of $>10^8$ pfu/ml in embryonated chicken eggs.

8. EXAMPLE 3

[0364] This example describes the production of nine-segmented influenza viruses based on the manipulation of the segment-specific packaging signals.

8.1 MATERIALS & METHODS

[0365] **Cells and viruses.** 293T cells were maintained in Dulbecco's modified Eagle's medium with 10 % fetal calf serum (FCS). MDCK cells were grown in Eagle's minimal essential medium with 10 % FCS. Viruses were grown in 10-day-old specific-pathogen-free chicken embryos at 37°C (Charles River Laboratories, SPAFAS).

[0366] **Plasmid construction.** (i) Generation of NA-PB1mut-NA, NA-PB2mut-NA, and NA-PAmut-NA constructs (Fig. 29A left). To introduce silent mutations at the two ends of each ORF, the ORFs of the PB1, PB2, and PA genes were amplified by PCR from previously constructed pDZ-PB1, PB2, and PA constructs (Quinlivan et al., 2005, J Virol 79:8431-9) and cloned into a pGEM-T vector (Promega). Primers (forward: 5'-ca gctagc atg gaC gtT aaC ccA acT CtG TtA ttT CtG aaG gtA ccG gcG caG aaC gcC atC agT acG acC ttT cct tat act gga gac-3' (SEQ ID NO:128); reverse: 5'-gt ctcgag cta Ctt Ctg TcT CcG Aag Ttc Ctc Gat Tgt ACT Gca Aat Ttt cat gat etc agt gaa c-3' (SEQ ID NO:129)) were used to amplify PB1mut ORF; Primers (forward: 5'-ca gctagc atg gaG CgG atC aaG gaG TtG CgG aaC TtG atg tcg cag tct cg cac-3' (SEQ ID NO:130); two reverse primers: 5'-tg TGA Atc Cgt CaA Gat AGA GCT Atc TcT Ctt TcT Ctt cat Cac TaG Tac cac gtc tcc ttg ccc-3' (SEQ ID NO:131) and 5'-ga ctcgag cta Gtt Aat Agc cat Acg Gat Cct Ctt Agt Tgc Cgt Ttg TGA Ate Cgt CaA G-3' (SEQ ID NO:132)) were used to amplify PB2mut ORF; Primers (forward: 5'-ca gctagc atg gaG gaC ttC gtA AgG caG tgT ttT aaC ccA atg atC gtT gaA ctC gcA gaG aaG acG atg aaG gag tat ggg gag g-3' (SEQ ID NO:133); reverse: 5'-gt ctcgag cta TGA TaG Cgc Gtg Cgt CaA Aaa Aga Att Aaa cca GCT Ggc Gtt aag caa aac cca g-3' (SEQ ID NO:134)) were used to amplify PAmut ORF. The capitalized letters in these primer sequences designate mutated nucleotides. Site-directed mutagenesis was used to remove one Nhe I site in PB1mut ORF (A1143G), and one Nhe I site in PAmut ORF (A1233G). The PB1mut, PB2mut and PAmut ORFs were subsequently used to replace the GFP ORF of previously constructed plasmid pDZ-GFP-2 using the Nhe I and Xho I sites (Gao et al., 2008, J Virol 82:6419-26), generating the NA-PB1mut-NA, NA-PB2mut-NA, and NA-PAmut-NA constructs (Fig. 29A). (ii) Generation of PB1-GFP-PB1, PB2-GFP-PB2, and PA-GFP-PA constructs (Fig. 29A right). The 2.7 kb Kpn I fragment from previously constructed pDZ-PB1 plasmid (Quinlivan et al., 2005, J Virol 79:8431-9) was transferred to the Kpn I site of the pUC18 vector and subjected to site-directed mutagenesis to mutate six ATGs (A25T, A29T, A71T, A119T, A142T, A146T), and to generate one Nhe I site (A148G, G151A, T152G) and one Xho I site (C2184T, A2185C). The 2.7 kb PB1 Kpn I fragment was then transferred back to the pDZ vector (Quinlivan et al., 2005, J Virol 79:8431-9) (in which the Nhe I and Xho I sites had been removed), resulting in a plasmid pDZ-PB1-ps. Following the same strategy, three ATGs (A28T, A58T, A109T) were mutated in the PB2 gene, and four mutations (C153G, C155T, T2175C, C2177A) were introduced to generate one Nhe I site and one Xho I site, resulting a plasmid pDZ-PB2-ps; six ATGs (A25T, A45T, A58T, A85T, A95T, A138T) were mutated in the PA gene, and five mutations (A142T, C143A, T144G, T2052C, A2055G) were introduced to generate one Nhe I site and one Xho I site, resulting a plasmid pDZ-PA-ps. The ORF of the GFP protein was digested from the pDZ-GFP-2 plasmid (Gao et al., 2008, J Virol 82:6419-26), and ligated to the Nhe I and Xho I sites of pDZ-PB1-ps, pDZ-PB2-ps and pDZ-PA-ps plasmids, respectively, generating the PB1-GFP-PB1, PB2-GFP-PB2, and PA-GFP-PA constructs (Fig. 29A). (iii) Generation of PB1-HA(HK)-PB1, PB2-HA(HK)-PB2 constructs (Fig. 30A). The A/HK/1/68 HA ORF was amplified by PCR from the pCAGGS-HK HA plasmid (Wang et al., 2009, PLoS Pathog 6:e1000796) using primers (forward: 5'-ca gctagc atg aag acc atc att get ttg age tac att ttc-3' (SEQ ID NO:135); reverse: 5'-gt ctcgag tca aat gca aat gtt gca cct aat gtt gcc tct c-3' (SEQ ID NO:136)). One internal Xho I site was deleted using site directed mutagenesis. The full length A/HK/1/68 HA ORF was then used to replace the GFP gene of the PB1-GFP-PB1 and PB2-GFP-PB2 constructs (Fig. 29A), generating the PB1-HA(HK)-PB1, PB2-HA(HK)-PB2 constructs (Fig. 30A). The GFP gene of the PB1-GFP-PB1 construct (Fig. 29A) was also replaced by a Renilla luciferase ORF amplified from the plasmid pRLtk

(Promega), generating the PB1-Luc-PB1 construct which was used to rescue the control virus - PB1(ps)+Luc (Fig. 31A). The nucleic acid sequences of the chimeric segments (in positive sense) generated are listed in Figure 32.

[0367] Reverse genetics for recombinant viruses. The method for generating recombinant influenza viruses was as described previously (Fodor et al., 1999, J Virol 73:9679-82, Gao et al., 2008, J Virol 82:6419-26; and Quinlivan et al., 2005, J Virol 79:8431-9).

[0368] Acrylamide gel electrophoresis of purified vRNA. The viruses were grown in 10-day-old eggs at 37°C and were subsequently processed by using a previously reported method (Gao et al., 2008, J Virol 82:6419-26). Briefly, virus was purified and RNA was isolated and run on a 2.8 % denaturing polyacrylamide gel which was then stained with a silver staining kit (Invitrogen).

[0369] Western blot. To detect the viral protein within virions, viruses [rA/PR/8/34, X31, -PB 1(ps)+HK HA and -PB2(ps)+HK HA] were grown in embyonated chicken eggs at 37 °C and concentrated through a 30% sucrose cushion. The pelleted virions were suspended in PBS and dissolved in 2x protein loading buffer (100 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate, 20% glycerol, 5% β-mercaptoethanol, and 0.2% bromophenol blue). To detect the expression of viral proteins in infected cells, 80% confluent MDCK cell monolayers in six-well dishes were infected with viruses [rA/PR/8/34, X31, -PB1(ps)+HK HA and -PB2(ps)+HK HA] at an moi of 10 to 0.0001. One day after infection, the cells were washed with PBS and harvested and lysed in 2x protein loading buffer. The protein lysates were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred onto a nitrocellulose membrane (Whatman, Inc.). The membrane was then probed with mouse monoclonal antibodies against A/PR/8/34 HA (PY102, 1:2,000 dilution) (Reale et al., 1986, J Immunol 137:1352-8), A/PR/8/34 NP (HT103, 1:1,000 dilution) (O'Neill et al., 1998, Embo J 17:288-96), A/HK/1/68 HA1 (66A6, 1:2,000 dilution) (Wang et al., 2009, PLoS Pathog 6:e1000796), and A/HK/1/68 HA2 (12D1, 1:2,000 dilution) (Wang et al., 2009, PLoS Pathog 6:e1000796).

[0370] Immunostaining of plaques. Previous methods were followed (Gao et al., 2008, J Virol 82:6419-26; Matrosovich et al., 2006, Virol J 3:63). A rabbit anti-A/PR/8/34 polyclonal antibody (1:2,000 dilution) was used for plaque visualization.

[0371] Viral growth kinetics. 10-day-old embryonated chicken eggs were inoculated with influenza viruses (100 PFU/egg) and incubated at 37°C. At 24, 48 and 72 hr post inoculation, the allantoic fluids were harvested and the titers of the viruses were determined by plaque assay or immunostaining of the plaques in MDCK cells. At each time point, three eggs were analyzed for each virus.

[0372] Mouse immunization and challenge. Eight-week-old female C57BL/6 mice (CRL) were anesthetized intraperitoneally with a mixture of ketamine and xylazine, and immunized intranasally with 50 μl of PBS or influenza viruses [-PB1(ps)+HK HA or - PB1(ps)+Luc, in a dose of 10^3 or 10^4 PFU/mouse]. The mice were monitored daily for weight loss over a 2-week period. Three weeks after immunization, mice were challenged by intranasal infection with either 100 mouse lethal dose 50 (MLD_{50}) of A/PR/8/34 or 33.3 MLD_{50} of X31 virus. Again mice were monitored daily for weight loss or other signs of disease over a 2-week period.

[0373] Hemagglutination inhibition (HI) assay. Blood samples were collected from mice prior to vaccination (at day 0) and prior to challenge (at day 21). Receptor destroying enzyme (Sigma) treatment was used to eliminate nonspecific inhibitors of hemagglutination. The protocols on "WHO manual on animal influenza diagnosis and surveillance" were followed (www.who.int).

[0374] H1/H3 sandwich ELISA. 96-well Immulon 2HB plates (NUNC) were coated with mouse anti-H3 HA monoclonal antibody 66A6 (IgG1) (Wang et al., 2009, PLoS Pathog 6:e1000796) (5μg/ml in PBS) by overnight incubation at 4°C. Plates were then blocked with 1% BSA in PBS at room temperature for 30 minutes. Two-fold dilutions of intact egg grown virus were added and plates were incubated for 3 hours at 37°C. The H1 subtype HA protein on captured virus particles was then probed with 1μg/ml anti-H1 HA antibody C179 (mouse IgG2a) (Okuno et al., 1993, J Virol 67:2552-8) for 3 hours at 37°C and detected by goat antimouse IgG2a-AP (Southern Biotech) (1:2000 dilution).

8.2 RESULTS

8.2.1. GENERATION OF RECOMBINANT A/PR/8/34 VIRUSES CARRYING A NINTH GFP SEGMENT

[0375] At restrictive temperature, a temperature sensitive influenza A virus has been shown capable of containing two sets of nonstructural protein (NS) segment-specific packaging signals located in two different segments: one set was derived from an NS segment that has a temperature sensitive defect in the NS1 gene and a second set was from the segment that encodes a wild type NS1 gene (Enami et al., 1991, Virology 185:291-8). To determine whether influenza A virus was able to incorporate two copies of NA segment-specific packaging sequences, the packaging signals of the PB1 segment were switched to those from the NA segment (Fig. 29A, left) while the original NA segment was unchanged. To accomplish this, the A/PR/8/34 PB1 ORF that carried serial synonymous mutations at the two ends, named PB1mut (Fig. 29A, left), was flanked by the NA segment-specific packaging sequences (including the 3' and 5' NCRs, as well as the terminal coding sequence of the NA ORF), thus generating the NA-PB1mut-NA segment (Fig. 29A, left). Based on

findings described herein and in Gao and Palese, 2009, Proc Natl Acad Sci USA 106:15891-6 that the partial packaging signals in the HA or NS ORF region can affect viral RNA incorporation, the two ends of the PB1 ORF were silently mutated. The synonymous mutations in the PB1mut ORF region include 24 nucleotides (nt) and 17 nt changes in the 3' and 5'-proximal regions, respectively. The ATGs in the 3' proximal NA region of the chimeric NA-PB1mut-NA segment were all mutated by site-directed mutagenesis so that translation would be initiated at the PB1mut gene start codon. Based on findings described herein and in Gao and Palese, 2009, Proc Natl Acad Sci USA 106:15891-6 for the HA and NS segments and data from other studies (Fujii et al., 2005, J Virol 79:3766-74; Gog et al., 2007, Nucleic Acids Res 35:1897-907; Hutchinson et al., 2008, J Virol 82:11869-79; Liang et al., 2008, J Virol 82:229-36; Marsh et al., 2007, J Virol 81:9727-36; and Marsh et al., 2008, J Virol 82:2295-304), it was surmised that this chimeric NA-PB1mut-NA construct in Fig. 29A would most likely utilize the flanking NA packaging signals due to the absence of proper PB1-specific packaging sequences.

[0376] Using reverse genetics, a -PB1(ps) virus that carries seven wild type A/PR/8/34 RNA segments (PB2, PA, HA, NP, NA, M, NS) and one chimeric NA-PB1mut-NA segment was successfully rescued (Fig. 29B). The -PB1(ps) virus was attenuated compared with wild type A/PR/8/34 virus, with lower titers in eggs and smaller plaques in MDCK cells (Fig. 29E & F). To determine whether the -PB1(ps) virus was able to incorporate a ninth segment that had PB1 segment-specific packaging signals, a PB1-GFP-PB1 construct was generated that carried 153 nt of PB1 packaging sequences in the 3' end and 159 nt in the 5' end (Fig. 29A, right). These 153 nt and 159 nt sequences consisted of both NCRs and terminal coding region packaging sequences and the six ATGs located in the 3' 153 nt PB1 packaging sequences were all mutated by site-directed mutagenesis. The - PB1(ps)+GFP virus that had all eight segments of the -PB1(ps) virus and a ninth GFP segment with PB1 segment-specific packaging signals (Fig. 29B) then was generated. - PB1(ps)+GFP virus exhibited similar growth characteristics to the -PB1(ps) virus, with similar titers in eggs and similar plaque phenotypes in MDCK cells (Fig. 29E & F). The - PB1(ps)+GFP virus was stable, and GFP expression in infected cells (Fig. 29G) was maintained over 5 passages in eggs by the limiting dilution technique. The percentage of GFP expressing plaques formed by the -PB1(ps)+GFP virus also did not change over 5 passages in eggs (Fig. 33).

[0377] Following the same strategy, the packaging signals of the PB2 and PA segments were also each replaced with those of NA. Chimeric constructs NA-PB2mut-NA and NA-PAmut-NA were generated (Fig. 29A, left). PB2mut ORF had 13 nt synonymous changes in the 3' end and 36 nt in the 5' end to inactivate the PB2 ORF region packaging signals; and PAmut ORF region carried 19 nt synonymous changes in the 3' end and the same number of changes in the 5' end to inactivate the PA ORF region packaging signals (Fig. 29A, left). The two chimeric GFP constructs PB2-GFP-PB2 and PA-GFP-PA that respectively carried PB2 and PA segment-specific packaging sequences were made using the same method utilized to produce the PB1-GFP-PB1 construct (Fig. 29A, right). The 3 ATGs in the 3' end 158 nt PB2 packaging sequences of the PB2-GFP-PB2, and 3 ATGs in the 3' end 129 nt PA packaging sequences of the PA-GFP-PA construct, were all mutated to TTGs in order for the GFP gene to utilize its own initiation codon (Fig. 29A, right). For the PB2 segment, a virus that has seven wild type A/PR/8/34 RNA segments (PB1, PA, HA, NP, NA, M, NS) and one chimeric segment NA-PB2mut-NA was not rescued. However, when a ninth PB2-GFP-PB2 construct was added, the -PB2(ps)+GFP virus was successfully rescued (Fig. 29C). The -PB2(ps)+GFP virus grew in eggs to a titer similar to that of the -PB1(ps)+GFP virus (Fig. 29E), but it produced slightly smaller plaques in MDCK cells (Fig. 29F). The expression of GFP in infected cells (Fig. 29G) and the percentage of GFP expressing plaques (Fig. 33) were also stably maintained over at least five passages in embryonated chicken eggs by the limiting dilution technique. For the PA segment, a -PA(ps) virus that has seven wild type A/PR/8/34 segments (PB2, PB1, HA, NP, NA, M, NS) and one chimeric segment NA-PAmut-NA (Fig. 29D) was successfully rescued. The -PA(ps)+GFP virus carrying the ninth PA-GFP-PA segment was also successfully rescued (Fig. 29D). The -PA(ps) and - PA(ps)+GFP viruses were more attenuated compared with the -PB1(ps), -PB1(ps)+GFP and the -PB2(ps)+GFP viruses, growing to lower titers in eggs and generating smaller plaques in MDCK cells (Fig. 29F). Due to small plaque size, the infectious titers of the -PA(ps) and - PA(ps)+GFP viruses could not be accurately measured and their growth rates in eggs was not further characterized. The GFP expression by the -PA(ps)+GFP virus in infected cells (Fig. 29G) was, however, stably maintained over at least five passages in embryonated chicken eggs. Finally, although the infectious titers of the -PB1(ps), -PB1(ps)+GFP and the - PB2(ps)+GFP viruses from eggs were much lower than that of recombinant (r)A/PR/8/34 virus (Fig. 29E), their hemagglutination assay titers were comparable to that of the rA/PR/8/34 virus two and three days post inoculation (Fig. 29H), suggesting that these viruses produced more defective virions than does the wild type virus. The number of synonymous mutations introduced to disrupt the packaging signals in the ORF region and the length of the flanking packaging sequences used in the chimeric constructs (Fig. 29A) were decided upon previous characterization of the A/WSN/33 viral RNA packaging signals (Fujii et al., 2003, Proc Natl Acad Sci U S A 100:2002-7; Liang et al., 2005, J Virol 79:10348-55; Liang et al., 2008, J Virol 82:229-36; Marsh et al., 2008, J Virol 82:2295-304; and Muramoto et al., 2006, J Virol 80:2318-25).

[0378] In conclusion, a novel approach to construct several nine-segmented influenza viruses simply by manipulating the RNA packaging sequences was generated. The resulting viruses were genetically stable and carried an extra GFP segment. Linearity between dilutions and plaque numbers was also observed for these nine-segmented viruses, suggesting indeed more than eight RNAs can be incorporated into one particle.

8.2.2. GENERATION OF RECOMBINANT INFLUENZA VIRUSES CARRYING BOTH A/PR/8/34(H1N1) AND A/HK/1/68(H3N2) HEMAGGLUTININS

[0379] Whether the method for generating the nine-segmented GFP virus could be used to generate influenza viruses coding for two subtypes of HA (the A/PR/8/34(H1N1) HA and the HA from A/HK/1/68(H3N2)) was determined. To do this, the GFP ORF regions of the PB1-GFP-PB1 and PB2-GFP-PB2 constructs (Fig. 29A, right) were each replaced by the A/HK/1/68 HA ORF, generating the PB1-HA(HK)-PB1 and PB2-HA(HK)-PB2 constructs (Fig. 30A). Using reverse genetics, two nine-segmented viruses named -PB1(ps)+HK HA (Fig. 2B) and -PB2(ps)+HK HA (Fig. 30C) were rescued. The -PB1(ps)+HK HA virus and the -PB2(ps)+HK HA virus had similar growth characteristics as the -PB1(ps)+GFP and the -PB2(ps)+GFP viruses (Figs. 29E & 30D), respectively. In order to show that both the A/PR/8/34 and the A/HK/1/68 HAs were incorporated into particles of the -PB1(ps)+HK HA and -PB2(ps)+HK HA viruses, four viruses [rA/PR/8/34, X31 that has six A/PR/8/34 internal genes and the A/HK/1/68 HA and NA genes, -PB2(ps)+HK HA and -PB 1(ps)+HK HA viruses] were grown in eggs and concentrated by passing through a sucrose cushion. Western blotting was then performed to detect the A/PR/8/34 and A/HK/1/68 HAs in purified virions (Fig. 30E). The results showed that when the same amounts of virus proteins were loaded, the -PB1(ps)+HK HA and -PB2(ps)+HK HA viruses had similar levels of A/PR/8/34 HA protein when compared with the wild type rA/PR/8/34 virus; this includes uncleaved HA0 and cleaved HA1 detected by the monoclonal mouse antibody (Mab) PY102 (Fig. 30E). Also, when comparable amounts of virus proteins were loaded, rA/PR/8/34 and X31 had the same amount of NP protein detected by Mab HT103 (Fig. 30E). However, for the -

[0380] PB1(ps)+HK HA and -PB2(ps)+HK HA chimeric viruses, the NP levels were about five times lower than those of rA/PR/8/34 and X31 viruses (Fig. 30E), indicating a less efficient RNP incorporation by the nine-segmented viruses. Both HA0 and HA1 from A/HK/1/68 were detected in the -PB1(ps)+HK HA and the -PB2(ps)+HK HA virus particles using Mab 66A6; notably, when normalized for total protein, H3 HA incorporation by the chimeric viruses was much lower than incorporation by the X31 virus, with lowest levels seen in the PB1(ps)+HK HA virus (Fig. 30E). The Western blot using Mab 12D1 to detect A/HK/1/68 HA0 and cleaved HA2 showed similar results (Fig. 30E). Western blotting then was used to detect the expression of both A/PR/8/34 and A/HK/1/68 HAs by the -PB 1(ps)+HK HA and - PB2(ps)+HK HA viruses in infected cells (Fig. 30F). Both A/PR/8/34 and A/HK/1/68 HAs were detected in MDCK cells infected by these viruses (Fig. 30F, lower panel). In contrast, as with Fig. 30E, cells infected with rA/PR/8/34 virus only expressed A/PR/8/34 HA and the X31 virus-infected cells only expressed H3 HA (Fig. 30F, upper panel).

[0381] Finally, a sandwich ELISA was performed to confirm that both H1 and H3 subtype HA proteins were incorporated into the nine-segmented virus particles (Fig. 30G). 96-well plates were coated with Mab 66A6 (Wang et al., 2009, PLoS Pathog 6:e1000796) to capture intact virus particles in an H3-dependent manner. Virus particles were then probed for H1 content with Mab C179, an antibody with activity against H1 and H2 subtype HA, but that does not react with H3 HA (Okuno et al., 1993, J Virol 67:2552-8). Signals were detected for the two nine-segmented viruses, indicating that indeed two types of HA proteins were incorporated into the virus particles. In contrast, both rA/PR/8/34 and X31 viruses gave negative results (Fig. 30G).

[0382] In conclusion, two recombinant viruses, each of which carried two subtypes of HA, one A/PR/8/34(H1N1) HA and one A/HK/1/68(H3N2) HA were successfully rescued. Both HAs were incorporated into virus particles and were expressed in virus infected MDCK cells.

[0383] To determine the RNA packaging efficiencies of the recombinant - PB1(ps)+HK HA and -PB2(ps)+HK HA viruses, RNA was isolated from the purified viruses and resolved on a 2.8% acrylamide gel followed by silver staining (Fig. 30H). The X31 virus has six A/PR/8/34 internal genes along with the A/HK/1/68 HA and NA segments which migrated to distinct positions from those of the A/PR/8/34 HA and NA (Fig. 30H). By comparing density of bands, it was observed that the -PB1(ps)+HK HA virus inefficiently incorporated the NA-PB1mut-NA segment. The PB1-HA(HK)-PB1 segment was also packaged somewhat inefficiently when compared with the A/PR/8/34 HA segment (Fig. 30H). For the -PB2(ps)+HK HA virus, the NA-PB2mut-NA segment was inefficiently packaged. In contrast, the PB2-HA(HK)-PB2 segment was packaged efficiently, with a level similar to that of A/PR/8/34 HA (Fig. 30H).

8.2.3. IMMUNIZATION OF MICE WITH A RECOMBINANT NINE-SEGMENTED VIRUS CONFERS PROTECTION FROM LETHAL CHALLENGES OF RA/PR/8/34 AND X31 VIRUSES

[0384] To test whether the nine-segmented influenza viruses carrying two subtypes of HA could be used as live vaccines, mouse challenge experiments were conducted. The - PB1(ps)+HK HA virus was arbitrarily chosen for the study. As a negative control immunogen the -PB1(ps)+Luc virus was used, which carries a ninth PB1-Luc-PB1 instead of a PB1-HA(HK)-PB1 segment (Fig. 30B and Fig. 34). Both -PB1(ps)+Luc and - PB1(ps)+HK HA viruses grew to similar titers as the -PB1(ps) virus in eggs (Fig. 31A). To test whether the nine-segmented viruses were pathogenic in mice, groups of eight-week-old female C57BL/6 mice were given PBS, -PB1(ps)+HK HA virus, or the -PB1(ps)+Luc virus, at either 10^3 or 10^4 PFU by intranasal administration (Fig. 31B). The mice infected with 10^4 PFU of either -PB1(ps)+Luc

or -PB1(ps)+HK HA virus died or lost more than 25% of their initial body weight by day eight post infection (Fig. 31B). The group of mice given 10^3 PFU of -PB1(ps)+Luc exhibited little or no weight loss and exhibited no signs of disease, similar to the PBS group (Fig. 31B). The group of mice given 10^3 PFU of -PB1(ps)+HK HA virus lost approximately 5% of their body weight by day seven post infection followed by full recovery within three days; no other signs of disease were observed (Fig. 31B). Since administration of 10^3 PFU of either chimeric virus caused very little or no changes associated with illness, exposure to this dose was considered to be analogous with vaccination.

[0385] Three weeks post infection, lethal virus challenge experiments were performed on the groups of mice infected with 10^3 PFU of -PB1(ps)+Luc virus, 10^3 PFU of - PB1(ps)+HK HA virus, or mice that were mock vaccinated with PBS. Mice were given 3,000 PFU (100 MLD_{50}) of rA/PR/8/34 virus by intranasal administration (Fig. 31C). In contrast to the PBS group, the groups vaccinated with either the PB1(ps)+Luc or the - PB1(ps)+HK HA viruses were completely protected from lethal challenge: no loss of body weight or signs of disease were observed (Fig. 31C). Following the same methods, 10^7 PFU (33 MLD_{50}) of X31 virus was administered intranasally to a second set of mice that were mock vaccinated (PBS group), vaccinated with 10^3 PFU -PB1(ps)+Luc, or vaccinated with 10^3 PFU -PB1(ps)+HK HA virus (Fig. 31D). The groups of mice that were mock or - PB1(ps)+Luc vaccinated quickly lost 25% of their body weight in three days and were sacrificed. Although previous findings showed that cellular responses to the internal NP and M proteins conferred some protection against heterologous challenges (Yewdell et al., 1985, Proc Natl Acad Sci USA 82:1785-9), no protection was observed in the -PB1(ps)+Luc vaccinated group possibly due to the high dosage of challenge virus used. In contrast, vaccination with 10^3 PFU of -PB1(ps)+HK HA virus protected the mice from the lethal challenge with X31 virus. Average body weight was reduced by 10% on the day following challenge and all mice quickly recovered (Fig. 31D).

[0386] Analysis of serum samples from this experiment indicated that by day 21 postvaccination all animals vaccinated with 10^3 PFU of -PB1(ps)+HK HA virus produced hemagglutination-inhibiting antibodies against rA/PR/8/34 virus, with titers ranging from 320 to 640. Four out of five animals produced low but detectable level of hemagglutination-inhibiting antibodies against X31 virus, with titers ranging from 20 to 40 (Table 17). As expected, animals vaccinated with 10^3 PFU of -PB1(ps)+Luc virus had only hemagglutination-inhibiting antibodies against rA/PR/8/34 virus, with titers ranging from 160 to 320 (Table 17). No hemagglutination-inhibiting antibodies against either rA/PR/8/34 or X31 virus were detected in serum from animals mock-vaccinated with PBS.

Table 17. Hemagglutination-inhibitory activity against rA/PR/8/34 and X31 viruses of sera from mice immunized with nine-segmented viruses.

		Titer against rA/PR/8/34		Titer against X31	
Vaccine	Mouse	Preimmune	Postvaccination	Preimmune	Postvaccination
PBS	1	<10	< 10	< 10	< 10
	2	<10	<10	< 10	< 10
	3	<10	<10	< 10	< 10
	4	<10	<10	< 10	< 10
	5	<10	<10	< 10	< 10
-PB 1 (ps) +Luc	1	< 10	160	< 10	< 10
	2	< 10	320	< 10	< 10
	3	< 10	160	< 10	< 10
	4	< 10	320	< 10	< 10
	5	< 10	320	< 10	< 10
-PB1(ps) +HK HA	1	< 10	320	< 10	20
	2	< 10	640	< 10	<10
	3	< 10	320	< 10	40
	4	< 10	320	< 10	20
	5	< 10	320	< 10	40

[0387] In conclusion, vaccination with 10 PFU of -PB1(ps)+HK HA virus was protective in mice against lethal challenge with influenza viruses from two separate subtypes: one H1N1 subtype (rA/PR/8/34) and one H3N2 subtype (X31).

8.3 DISCUSSION

[0388] Two recombinant viruses were generated, named -PB1(ps) (Fig. 29B) and - PA(ps) (Fig. 29D) which lacked either PB1 or PA packaging sequences, respectively, and carried NA packaging sequences in their place. These viruses

were viable, however, both the PB 1 and the PA packaging signals were important for virus growth since the replacement of the PB1 segment by NA-PB1mut-NA, or the PA segment by NA-PAmut-NA did have a significant effect on the packaging of both chimeric segments (Fig. 30H) as well as on virus growth rates (Fig. 29E, F). The ability to rescue both viruses might indicate that influenza genomic RNA packaging does not absolutely require PB1 or PA packaging signals. Based on findings of packaging of the HA and NS segments described herein and in Gao and Palese, 2009, Proc Natl Acad Sci USA 106:15891-6, it was hypothesized that the two chimeric segments, NA-PB1mut-NA and NA-PAmut-NA (Fig. 29A, left), would likely utilize the flanking NA packaging signals instead of the PB 1 and PA packaging signals, respectively. However, it is possible that the PB1 or PA ORF region carrying the serial synonymous mutations (Fig. 29A) partially retained the PB1 or PA packaging signals. Although 24 and 17 nt changes were introduced to the PB1 ORF and two sets of 19 nt changes were made in the PA ORF (Fig. 29A, left), some residual PB1 or PA packaging signals could still exist, enabling PB1 or PA segment-specific recognition (Fig. 29A, left). Interestingly, both viruses were able to incorporate a ninth segment coding for GFP. When supplied with a ninth PB1-GFP-PB1 segment (Fig. 29A, right) flanked by the PB1 packaging sequence, the -PB1(ps) virus was able to stably incorporate it into the virus genome, generating the -PB1(ps)+GFP virus (Fig. 29B); likewise, the -PA(ps)+GFP virus was able to maintain an extra PA-GFP-PA segment flanked by the PA packaging signals (Fig. 29D). The generation of both viruses with an extra GFP segment reflected the tendency of influenza virus to have a complete set of packaging signals on its genomic RNAs.

[0389] For the PB2 segment, when the wild type PB2 was replaced by the NA-PB2mut-NA chimeric segment (Fig. 29A, left), the virus could not be rescued. This was also seen in previous studies using A/WSN/33 virus in which mutating or deleting the PB2 packaging sequences resulted in a more severe packaging defect than did manipulation of other segments (Liang et al., 2008, J Virol 82:229-36; Muramoto et al., 2006, J Virol 80:2318-25). However, when a ninth PB2-GFP-PB2 segment that carried PB2 packaging signals was included (Fig. 29A, right), the -PB2(ps)+GFP virus was successfully rescued (Fig. 29C). This result also reflected the preference of influenza virus to carry sets of eight unique packaging signals.

[0390] Using the strategy that was designed for generation of the -PB1(ps)+GFP (Fig. 29B) and -PB2(ps)+GFP (Fig. 29C) viruses, two recombinant viruses were rescued that encoded two different full length HAs: both -PB1(ps)+HK HA virus (Fig. 2B) and - PB2(ps)+HK HA virus (Fig. 2C) encoded an A/PR/8/34 HA and an A/HK/1/68 HA. Thus, a novel approach to engineer viruses encoding two different HAs was generated. These viruses are significantly attenuated compared to the wild type virus, with lower growth rates in eggs and smaller plaques in MDCK cells (Figs. 29 & 30). The MLD_{50} of -PB1(ps)+HK HA was between 10^3 and 10^4 PFU (Fig. 31B), significantly higher than that of wild type A/PR/8/34 virus, which has an MLD_{50} of about 30 PFU. Immunization of mice with 1000 PFU of - PB1(ps)+HK HA virus completely protected them from the lethal challenge with rA/PR/8/34 virus or X31 virus, suggesting that this nine-segmented virus strategy might be utilized for the development of bivalent live attenuated influenza vaccines. Although the -PB1(ps)+HK HA virus is potentially lethal to mice, a similar approach can be applied to other less virulent viruses for a live vaccine purpose. Current seasonal influenza vaccines must include three distinct influenza viruses: one A (H3N2) virus, one regular seasonal A (H1N1) virus, and one B virus. The bivalent, nine-segmented influenza viruses described herein offer a means of combining two major antigens (e.g. H1 and H3 HAs) into one vaccine strain. This may be particular useful if the number of co-circulating influenza virus lineages increases to more than three: for example, in 2009, a novel swine origin influenza A virus of the H1N1 subtype, which is different from seasonal H1N1 virus, emerged from North America and caused an influenza pandemic. Furthermore, by carrying specific antigens on its ninth chimeric segment, this nine-segmented influenza virus platform could also be applied to generate vaccines against other bacterial or viral pathogens.

9. EXAMPLE 4

[0391] This example demonstrates how reassortment of viruses can be measured.

[0392] A reverse genetics approach can be used to assess whether each of the chimeric gene segments of the recombinant influenza viruses shown in, e.g., Figures 35 to 37, can reassort. Cells expressing the necessary influenza virus proteins can be co-transfected with influenza virus chimeric segments that have had their packaging signals swapped and influenza virus gene segments from a wild-type or lab strain of influenza virus, wherein the wild-type or lab strain influenza virus gene segments include a gene segment that encodes an influenza virus protein encoded by one of the chimeric influenza virus gene segments and the other gene segments necessary to produce a replication-competent influenza virus. For example, cells, such as 293T cells, MDCK cells or Vero cells, expressing the necessary viral proteins (*e.g.*, PA, PB1, PB2, and NP) can be transfected with plasmids encoding four of the chimeric gene segments shown in Figure 35 (NA-PB2mut-NA, PB2-PB1mut-PB2, PB1-PAmut-PB1, and PA-NAmut-PA) and plasmids encoding five gene segments (pDZ-NP, NA, M, NS, and HA) of a wild-type influenza virus or a lab strain, such as A/PR/8/34, using techniques previously described (see, *e.g.,* Gao et al., 2008, J. Virol. 82: 6419-6426; Quinlivan et al., 2005, J. Virol. 79: 8431-8439; Fodor et al., 1999, J. Virol. 73: 9679-9682). The recombinant viruses rescued can then be grown in tissue culture or embryonated eggs and plaque purified using known techniques. The gene segments present in the plaque purified viruses can then be determined by, e.g., amplifying single plaques, isolating the vRNA from the virus, subjecting

the vRNA to RT-PCR using primers designed to hybridize to specific gene segments and running the RT-PCR products on an agarose gel. Alternatively, the vRNA segments from the plaque performed viruses can be sequenced using techniques known in the art, such as deep sequencing. The inability to detect influenza viruses containing less than the combination of the chimeric gene segments that have had their packaging signals swapped indicates that those chimeric gene segments are unable to reassort freely. For example, with respect to the chimeric gene segments of the recombinant virus shown in Figure 35, the inability to detect influenza viruses containing the three chimeric NA-PB2mut-NA, PB2-PB1mut-PB2, and PB1-PAmut-PB1 gene segments and the wild-type or lab strain influenza virus NA, NP, M, NS and HA gene segments indicates that the four chimeric gene segments (NA-PB2mut-NA, PB2-PB1mut-PB2, PB1-PAmut-PB1, and PA-NAmut-PA) are unable to reassort freely.

[0393] As another approach to determine whether the chimeric gene segments of the recombinant influenza viruses shown in, e.g., Figures 35 to 37 can freely reassort in tissue culture, cells (*e.g.,* 293T cells, MDCK cells or Vero cells) can be co-infected with the recombinant virus shown in, *e.g.,* Figure 35, 36 or 37, and a wild-type or lab strain of influenza virus at certain multiplicity of infection ("moi") for each virus (*e.g.,* an moi of 10). The resulting viruses can then be plaque purified. The gene segments present in the plaque purified viruses can then be determined by, *e.g.,* amplifying single plaques, isolating the vRNA from the virus, subjecting the vRNA to RT-PCR using primers designed to hybridize to specific gene segments and running the RT-PCR products on an agarose gel. Alternatively, the vRNA segments from the plaque performed viruses can be sequenced using techniques known in the art, such as deep sequencing. The inability to detect viruses containing less than the combination of the chimeric segments that have had their packaging signals swapped are unable to reassort freely. For example, with respect to the chimeric gene segments of the recombinant virus shown in Figure 35, the inability to detect influenza viruses containing the three chimeric NA-PB2mut-NA, PB2-PB1mut-PB2, and PB1-PAmut-PB1 gene segments and the wild-type or lab strain influenza virus NA, NP, M, NS and HA gene segments indicates that the four chimeric gene segments (NA-PB2mut-NA, PB2-PB1mut-PB2, PB1-PAmut-PB1, and PA-NAmut-PA) are unable to reassort freely.

10. EXAMPLE 5

[0394] This example describes the production of a nine segment recombinant influenza virus.

[0395] A chimeric construct designated PA-NAmut-PA was generated as follows: the A/PR/8/34 NA ORF that carries silent mutations at the two ends, named NAmut, was ligated to the A/PR/8/34 PA packaging sequences, generating the PA-NAmut-PA construct. A chimeric construct designated NA-GFP-NA was generated as follows: a GFP ORF was ligated to the A/PR/8/34 NA packaging sequence, generating the NA-GFP-NA construct. A chimeric construct designated NA-HA(HK)-NA was generated as follows: the HA ORF from the A/Hong Kong/1/68 (A/HK/1/68) HA gene was ligated to the A/PR/8/34 NA packaging sequences, generating the NA-HA(HK)-NA construct. (See Fig. 38.)

[0396] Recombinant influenza viruses (see Fig. 38) were generated using a method modified Example 1 and from Gao and Palese, 2009, PNAS 106:15891. 293T cells were transfected with 2 chimeric plasmids [PA-NAmut-PA and NA-GFP-NA or NA-HA(HK)-NA], and 7 plasmids carrying the wild type A/PR/8/34 PB2, PB1, PA, HA, NP, M, NS segments. 24 hours post transfection, the cells were harvested and inoculated into 10-day-old specific-pathogen-free chicken embryos (Charles River Laboratories, SPAFAS, Preston, CT). Three days later, the allantoic fluids were harvested and HA assay was used to determine the existence of rescued virus. The virus titers were determined by plaque assay in MDCK cells. This 9-segment chimeric virus grew well, with titers of $>10^8$ pfu/ml in embryonated chicken eggs.

[0397] The invention is not to be limited in scope by the specific embodiments described herein.

Claims

1. A recombinant influenza A, B, or C virus, comprising influenza virus gene segments, wherein at least two of the influenza virus gene segments are chimeric influenza virus gene segments, wherein:

   (a) a first chimeric influenza virus gene segment comprises, in the order presented:

   (i) packaging signals found in the 3' non-coding region of a first influenza virus gene segment;
   (ii) packaging signals found in the 3' proximal coding region of the first influenza virus gene segment, wherein the 3' proximal coding region of the first influenza virus gene segment has been mutated to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence;
   (iii) an open reading frame from a second influenza virus gene segment, wherein the open reading frame contains one, two, three or more silent mutations in the virus packaging signals found in the 3' and 5' proximal nucleotides in the open reading frame;
   (iv) packaging signals found in the 5' proximal coding region of the first influenza virus gene segment; and

(v) packaging signals found in the 5' non-coding region of the first influenza virus gene segment; and

(b) a second chimeric influenza virus gene segment comprises, in the order presented:

(i) packaging signals found in the 3' non-coding region of the second influenza virus gene segment;
(ii) packaging signals found in the 3' proximal coding region of the second influenza virus gene segment, wherein the 3' proximal coding region of the second influenza virus gene segment has been mutated to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence;
(iii) an open reading frame from the first influenza virus gene segment, wherein the open reading frame contains one, two, three or more silent mutations in the virus packaging signals found in the 3' and 5' proximal nucleotides in the open reading frame;
(iv) packaging signals found in the 5' proximal coding region of the second influenza virus gene segment; and
(v) packaging signals found in the 5' non-coding region of the second influenza virus gene segment;

wherein the first and second gene segments are two different gene segments, and wherein the recombinant influenza virus has a reduced ability to reassort with a different influenza virus such that the progeny influenza virus with the combined gene segments has reduced replication competence, wherein a virus with reduced replication competence is a virus that produces at least 1 log, 1.5 logs, 2 logs, 25 logs, 3 logs, 3.5 logs, 4 logs, 4.5 logs, 5 logs, 5.5 logs, 6 logs, 6.5 logs, 7 logs, 7.5 logs, 8 logs, 8.5 logs, 9 logs or 10 logs lower titers of replicating progeny than the replicating progeny produced by a wild-type influenza virus of the same type.

2. A recombinant influenza A, B, or C virus, comprising influenza virus gene segments, wherein at least three of the influenza virus gene segments are chimeric influenza virus gene segments, wherein:

(a) a first chimeric influenza virus gene segment comprises, in the order presented:

(i) packaging signals found in the 3' non-coding region of a third influenza virus gene segment;
(ii) packaging signals found in the 3' proximal coding region of the third influenza virus gene segment, wherein the 3' proximal coding region of the third influenza virus gene segment has been mutated to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence;
(iii) an open reading frame from a first influenza virus gene segment, wherein the open reading frame contains one, two, three or more silent mutations in the virus packaging signals found in the 3' and 5' proximal nucleotides in the open reading frame;
(iv) packaging signals found in the 5' proximal coding region of the third influenza virus gene segment; and
(v) packaging signals found in the 5' non-coding region of the third influenza virus gene segment; and

(b) a second chimeric influenza virus gene segment comprises, in the order presented:

(i) packaging signals found in the 3' non-coding region of the first influenza virus gene segment;
(ii) packaging signals found in the 3' proximal coding region of the first influenza virus gene segment, wherein the 3' proximal coding region of the first influenza virus gene segment has been mutated to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence;
(iii) an open reading frame from a second influenza virus gene segment, wherein the open reading frame contains one, two, three or more silent mutations in the virus packaging signals found in the 3' and 5' proximal nucleotides in the open reading frame;
(iv) packaging signals found in the 5' proximal coding region of the first influenza virus gene segment; and
(v) packaging signals found in the 5' non-coding region of the first influenza virus gene segment; and

(c) a third chimeric influenza virus gene segment comprising, in the order presented:

(i) packaging signals found in the 3' non-coding region of the second influenza virus gene segment;
(ii) packaging signals found in the 3' proximal coding region of the second influenza virus gene segment, wherein the 3' proximal coding region of the second influenza virus gene segment has been mutated to eliminate any start codons and preclude the translation of the 3' proximal coding region sequence;
(iii) an open reading frame from the third influenza virus gene segment, wherein the open reading frame contains one, two, three or more silent mutations in the virus packaging signals found in the 3' and 5' proximal nucleotides in the open reading frame;
(iv) packaging signals found in the 5' proximal coding region of the second influenza virus gene segment; and

(v) packaging signals found in the 5' non-coding region of the second influenza virus gene segment;
wherein the first, second and third gene segments are three different gene segments, and
wherein the recombinant influenza virus has a reduced ability to reassort with a different influenza virus such that the progeny influenza virus with the combined gene segments has reduced replication competence, wherein a virus with reduced replication competence is a virus that produces at least 1 log, 1.5 logs, 2 logs, 25 logs, 3 logs, 3.5 logs, 4 logs, 4.5 logs, 5 logs, 5.5 logs, 6 logs, 6.5 logs, 7 logs, 7.5 logs, 8 logs, 8.5 logs, 9 logs or 10 logs lower titers of replicating progeny than the replicating progeny produced by a wild-type influenza virus of the same type.

3. The recombinant influenza virus of claim 1 or 2, wherein the first, second or third influenza virus gene segment refers to an HA, NA, NS, PB1, PB2, PA, M, or NP gene segment from an influenza virus.

4. The recombinant influenza virus of claim 1, wherein the first chimeric influenza virus gene segment encodes HA protein and the second chimeric influenza virus gene segment encodes NA protein.

5. The recombinant influenza virus of claim 4, wherein the second chimeric influenza virus segment comprises:

(a) the 3' non-coding region nucleotide sequence consisting of SEQ ID NO: 19 and the 3' proximal coding region sequence consisting of SEQ ID NO: 20, and
(b) the 5' non-coding region nucleotide sequence consisting of SEQ ID NO: 22 and the 5' proximal coding region sequence consisting of SEQ ID NO: 23.

6. The recombinant influenza virus of claim 4 or 5, wherein the first chimeric influenza virus gene segment comprises:

(a) the 3' non-coding region nucleotide sequence consisting of SEQ ID NO: 31 and the 3' proximal coding region sequence consisting of SEQ ID NO: 32, and
(b) the 5' non-coding region nucleotide sequence consisting of SEQ ID NO: 34 and a 5' proximal coding region sequence consisting of SEQ ID NO: 35.

7. The recombinant influenza virus of claim 2, wherein the first, second, and third influenza virus gene segments encode the HA, NA, and NS proteins, respectively.

8. The recombinant influenza virus of claim 1, wherein

(a) the first chimeric influenza virus gene segment encodes NS protein, and wherein the 3' proximal coding region is mutated so as to eliminate the mRNA 5' splice site; or
(b) the first chimeric influenza virus gene segment encodes M protein, and wherein the 3' proximal coding region is mutated so as to eliminate the mRNA 5' splice site.

9. The recombinant influenza virus of any one of claims 1 to 8, wherein the recombinant influenza virus reassorts with other influenza viruses by less than 5%, as determined by the percentage of viral plaques containing reassorted influenza viruses with one or more chimeric influenza virus gene segments that have reassorted independently from one or more other chimeric influenza virus gene segments.

10. The recombinant influenza virus of any one of claims 1 to 9, wherein the virus is attenuated.

11. A host comprising the recombinant influenza virus of any one of claims 1 to 10, wherein the host is a chicken or avian cell, a cell from a cell line, or a chicken or avian embryonated egg.

12. A pharmaceutical composition or an immunogenic composition comprising the recombinant influenza virus of any one of claims 1 to 10.

13. The recombinant influenza virus of any one of claims 1 to 10, or the composition of claim 12, for use in eliciting an immune response against an influenza virus in a subj ect.

14. The recombinant influenza virus of any one of claims 1 to 10, or the composition of claim 12, for use in preventing or treating an influenza virus disease in a subject.

15. The recombinant influenza virus of any one of claims 1 to 10, or the composition of claim 12, for use in treating an influenza virus infection in a subject.

16. The recombinant influenza virus for use according to any one of claims 13 to 15, wherein the subject is a human.

17. An *in vitro* method for propagating a recombinant influenza virus, wherein the method comprises infecting a chicken or avian cell or a cell from a cell line with the recombinant influenza virus of any one of claims 1 to 10; and purifying the virus subsequently from said cell.

18. An *in ovo* method for propagating a recombinant influenza virus, wherein the method comprises infecting a chicken or avian embryonated egg with the recombinant influenza virus of any one of claims 1 to 10; and purifying the virus subsequently from said egg.

Patentansprüche

1. Rekombinantes Influenzavirus A, B oder C, umfassend Influenzavirus-Gensegmente, wobei mindestens zwei der Influenzavirus-Gensegmente chimäre Influenzavirus-Gensegmente sind, wobei:

(a) ein erstes chimäres Influenzavirus-Gensegment, in der präsentierten Reihenfolge, umfasst:

(i) Verpackungssignale aus der 3'-nichtcodierenden Region eines ersten Influenzavirus-Gensegments;
(ii) Verpackungssignale aus der proximalen 3'-codierenden Region des ersten Influenzavirus-Gensegments, wobei die proximale 3'-codierende Region des ersten Influenzavirus-Gensegments mutiert worden ist, um jegliche Start-Codons zu eliminieren und die Translation der Sequenz der proximalen 3'-codierenden Region auszuschließen;
(iii) einen offenen Leserahmen aus einem zweiten Influenzavirus-Gensegment, wobei der offene Leserahmen eine, zwei, drei oder mehr stille Mutationen in den Virusverpackungssignalen aus den proximalen 3'- und 5'-Nukleotiden in dem offenen Leserahmen enthält;
(iv) Verpackungssignale aus der proximalen 5'-codierenden Region des ersten Influenzavirus-Gensegments; und
(v) Verpackungssignale aus der 5'-nichtcodierenden Region des ersten Influenzavirus-Gensegments; und

(b) ein zweites chimäres Influenzavirus-Gensegment, in der präsentierten Reihenfolge, umfasst:

(i) Verpackungssignale aus der 3'-nichtcodierenden Region des zweiten Influenzavirus-Gensegments;
(ii) Verpackungssignale aus der proximalen 3'-codierenden Region des zweiten Influenzavirus-Gensegments, wobei die proximale 3'-codierenden Region des zweiten Influenzavirus-Gensegments mutiert worden ist, um jegliche Start-Codons zu eliminieren und die Translation der Sequenz der proximalen 3'-codierenden Region auszuschließen;
(iii) einen offenen Leserahmen aus dem ersten Influenzavirus-Gensegment, wobei der offene Leserahmen eine, zwei, drei oder mehr stille Mutationen in den Virusverpackungssignalen aus den proximalen 3'- und 5'-Nukleotiden in dem offenen Leserahmen enthält;
(iv) Verpackungssignale aus der proximalen 5'-codierenden Region des zweiten Influenzavirus-Gensegments; und
(v) Verpackungssignale aus der 5'-nichtcodierenden Region des zweiten Influenzavirus-Gensegments;
wobei das erste und zweite Gensegment zwei unterschiedliche Gensegmente sind, und
wobei das rekombinante Influenzavirus eine reduzierte Befähigung aufweist, sich mit einem unterschiedlichen Influenzavirus neu zu sortieren, sodass die Influenzavirusnachkommen mit den kombinierten Gensegmenten reduzierte Replikationsfähigkeit aufweisen, wobei ein Virus mit reduzierter Replikationsfähigkeit ein Virus ist, das mindestens 1 log, 1,5 logs, 2 logs, 25 logs, 3 logs, 3,5 logs, 4 logs, 4,5 logs, 5 logs, 5,5 logs, 6 logs, 6,5 logs, 7 logs, 7,5 logs, 8 logs, 8,5 logs, 9 logs oder 10 logs niedrigere Titer an replizierenden Nachkommen produziert als die von einem Wildtyp-Influenzavirus der gleichen Art produzierten replizierenden Nachkommen.

2. Rekombinantes Influenzavirus A, B oder C, umfassend Influenzavirus-Gensegmente, wobei mindestens drei der Influenzavirus-Gensegmente chimäre Influenzavirus-Gensegmente sind, wobei:

(a) ein erstes chimäres Influenzavirus-Gensegment, in der präsentierten Reihenfolge, umfasst:

(i) Verpackungssignale aus der 3'-nichtcodierenden Region eines dritten Influenzavirus-Gensegments;
(ii) Verpackungssignale aus der proximalen 3'-codierenden Region des dritten Influenzavirus-Gensegments, wobei die proximale 3'-codierende Region des dritten Influenzavirus-Gensegments mutiert worden ist, um jegliche Start-Codons zu eliminieren und die Translation der Sequenz der proximalen 3'-codierenden Region auszuschließen;
(iii) einen offenen Leserahmen aus einem ersten Influenzavirus-Gensegment, wobei der offene Leserahmen eine, zwei, drei oder mehr stille Mutationen in den Virusverpackungssignalen aus den proximalen 3'- und 5'-Nukleotiden in dem offenen Leserahmen enthält;
(iv) Verpackungssignale aus der proximalen 5'-codierenden Region des dritten Influenzavirus-Gensegments; und
(v) Verpackungssignale aus der 5'-nichtcodierenden Region des dritten Influenzavirus-Gensegments; und

(b) ein zweites chimäres Influenzavirus-Gensegment, in der präsentierten Reihenfolge, umfasst:

(i) Verpackungssignale aus der 3'-nichtcodierenden Region des ersten Influenzavirus-Gensegments;
(ii) Verpackungssignale aus der proximalen 3'-codierenden Region des ersten Influenzavirus-Gensegments, wobei die proximale 3'-codierende Region des ersten Influenzavirus-Gensegments mutiert worden ist, um jegliche Start-Codons zu eliminieren und die Translation der Sequenz der proximalen 3'-codierenden Region auszuschließen;
(iii) einen offenen Leserahmen aus einem zweiten Influenzavirus-Gensegment, wobei der offene Leserahmen eine, zwei, drei oder mehr stille Mutationen in den Virusverpackungssignalen aus den proximalen 3'- und 5'-Nukleotiden in dem offenen Leserahmen enthält;
(iv) Verpackungssignale in der proximalen 5'-codierenden Region des ersten Influenzavirus-Gensegments; und
(v) Verpackungssignale aus der 5'-nichtcodierenden Region des ersten Influenzavirus-Gensegments; und

(c) ein drittes chimäres Influenzavirus-Gensegment, umfassend in der präsentierten Reihenfolge:

(i) Verpackungssignale aus der 3'-nichtcodierenden Region des zweiten Influenzavirus-Gensegments;
(ii) Verpackungssignale aus der proximalen 3'-codierenden Region des zweiten Influenzavirus-Gensegments, wobei die proximale 3'-codierenden Region des zweiten Influenzavirus-Gensegments mutiert worden ist, um jegliche Start-Codons zu eliminieren und die Translation der Sequenz der proximalen 3'-codierenden Region auszuschließen;
(iii) einen offenen Leserahmen aus dem dritten Influenzavirus-Gensegment, wobei der offene Leserahmen eine, zwei, drei oder mehr stille Mutationen in den Virusverpackungssignalen aus den proximalen 3'- und 5'-Nukleotiden in dem offenen Leserahmen enthält;
(iv) Verpackungssignale aus der proximalen 5'-codierenden Region des zweiten Influenzavirus-Gensegments; und
(v) Verpackungssignale aus der 5'-nichtcodierenden Region des zweiten Influenzavirus-Gensegments;
wobei das erste, zweite und dritte Gensegment drei unterschiedliche Gensegmente sind, und
wobei das rekombinante Influenzavirus eine reduzierte Befähigung aufweist, sich mit einem unterschiedlichen Influenzavirus neu zu sortieren, sodass die Influenzavirusnachkommen mit den kombinierten Gensegmenten reduzierte Replikationsfähigkeit aufweisen, wobei ein Virus mit reduzierter Replikationsfähigkeit ein Virus ist, das mindestens 1 log, 1,5 logs, 2 logs, 25 logs, 3 logs, 3,5 logs, 4 logs, 4,5 logs, 5 logs, 5,5 logs, 6 logs, 6,5 logs, 7 logs, 7,5 logs, 8 logs, 8,5 logs, 9 logs oder 10 logs niedrigere Titer an replizierenden Nachkommen produziert als die von einem Wildtyp-Influenzavirus der gleichen Art produzierten replizierenden Nachkommen.

3. Rekombinantes Influenzavirus nach Anspruch 1 oder 2, wobei das erste, zweite oder dritte Influenzavirus-Gensegment ein HA-, NA-, NS-, PB1-, PB2-, PA-, M- oder NP-Gensegment aus einem Influenzavirus ist.

4. Rekombinantes Influenzavirus nach Anspruch 1, wobei das erste chimäre Influenzavirus-Gensegment HA-Protein codiert und das zweite chimäre Influenzavirus-Gensegment NA-Protein codiert.

5. Rekombinantes Influenzavirus nach Anspruch 4, wobei das zweite chimäre Influenzavirus-Gensegment umfasst:

(a) die Nukleotidsequenz der 3'-nichtcodierenden Region, bestehend aus SEQ ID NO: 19, und die Sequenz der proximalen 3'-codierenden Region, bestehend aus SEQ ID NO: 20, und
(b) die Nukleotidsequenz der 5'-nichtcodierenden Region, bestehend aus SEQ ID NO: 22, und die Sequenz der proximalen 5'-codierenden Region, bestehend aus SEQ ID NO: 23.

6. Rekombinantes Influenzavirus nach Anspruch 4 oder 5, wobei das erste chimäre Influenzavirus-Gensegment umfasst:

(a) die Nukleotidsequenz der 3'-nichtcodierenden Region, bestehend aus SEQ ID NO: 31, und die Sequenz der proximalen 3'-codierenden Region, bestehend aus SEQ ID NO: 32, und
(b) die Nukleotidsequenz der 5'-nichtcodierenden Region, bestehend aus SEQ ID NO: 34, und eine Sequenz der proximalen 5'-codierenden Region, bestehend aus SEQ ID NO: 35.

7. Rekombinantes Influenzavirus nach Anspruch 2, wobei das erste, zweite und dritte Influenzavirus-Gensegment jeweils die HA-, NA- und NS-Proteine codieren.

8. Rekombinantes Influenzavirus nach Anspruch 1, wobei

(a) das erste chimäre Influenzavirus-Gensegment NS-Protein codiert und wobei die proximale 3'-codierende Region mutiert ist, um die 5'-mRNA-Spleißstelle zu eliminieren; oder
(b) das erste chimäre Influenzavirus-Gensegment M-Protein codiert und wobei die proximale 3'-codierende Region mutiert ist, um die 5'-mRNA-Spleißstelle zu eliminieren.

9. Rekombinantes Influenzavirus nach einem der Ansprüche 1 bis 8, wobei sich das rekombinante Influenzavirus mit weiteren Influenzaviren um weniger als 5 % neu sortiert, wie durch den Prozentsatz von viralen Plaques, die neu sortierte Influenzaviren mit einem oder mehreren chimären Influenza-Gensegmenten enthalten, die sich unabhängig von einem oder mehreren weiteren chimären Influenzavirus-Gensegmenten neu sortiert haben, bestimmt.

10. Rekombinantes Influenzavirus nach einem der Ansprüche 1 bis 9, wobei das Virus abgeschwächt ist.

11. Wirt, umfassend das rekombinante Influenzavirus nach einem der Ansprüche 1 bis 10, wobei der Wirt eine Hühner- oder Vogelzelle, eine Zelle aus einer Zelllinie oder ein embryoniertes Hühner- oder Vogelei ist.

12. Pharmazeutische Zusammensetzung oder eine immunogene Zusammensetzung, umfassend das rekombinante Influenzavirus nach einem der Ansprüche 1 bis 10.

13. Rekombinantes Influenzavirus nach einem der Ansprüche 1 bis 10 oder die Zusammensetzung nach Anspruch 12, zur Verwendung beim Hervorrufen einer Immunantwort gegen ein Influenzavirus bei einem Subjekt.

14. Rekombinantes Influenzavirus nach einem der Ansprüche 1 bis 10 oder die Zusammensetzung nach Anspruch 12, zur Verwendung beim Verhindern oder Behandeln einer Influenzaviruserkrankung bei einem Subjekt.

15. Rekombinantes Influenzavirus nach einem der Ansprüche 1 bis 10 oder die Zusammensetzung nach Anspruch 12, zur Verwendung beim Behandeln einer Virusinfektion bei einem Subjekt.

16. Rekombinantes Influenzavirus nach einem der Ansprüche 13 bis 15, wobei das Subjekt ein Mensch ist.

17. In-vitro-Verfahren zur Vermehrung eines rekombinanten Influenzavirus, wobei das Verfahren Infizieren einer Hühner- oder Vogelzelle oder einer Zelle aus einer Zelllinie mit dem rekombinanten Influenzavirus nach einem der Ansprüche 1 bis 10; und nachfolgendes Reinigen des Virus von der Zelle umfasst.

18. *In-ovo*-Verfahren zur Vermehrung eines rekombinanten Influenzavirus, wobei das Verfahren Infizieren eines embryonierten Hühner- oder Vogeleis mit dem rekombinanten Influenzavirus nach einem der Ansprüche 1 bis 10; und nachfolgendes Reinigen des Virus von dem Ei umfasst.

Revendications

1. Virus de la grippe A, B ou C recombinant, comprenant des segments de gène de virus de la grippe, dans lequel au moins deux des segments de gène de virus de la grippe sont des segments de gène de virus de la grippe chimériques, dans lequel :

(a) un premier segment de gène de virus de la grippe chimérique comprend, dans l'ordre présenté :

(i) des signaux d'encapsidation trouvés dans la région non codante 3' d'un premier segment de gène de virus de la grippe ;
(ii) des signaux d'encapsidation trouvés dans la région codante proximale 3' du premier segment de gène de virus de la grippe, dans lequel la région codante proximale 3' du premier segment de gène de virus de la grippe a été mutée pour éliminer tout codon d'initiation et empêcher la traduction de la séquence de la région codante proximale 3' ;
(iii) un cadre de lecture ouvert d'un deuxième segment de gène de virus de la grippe, dans lequel le cadre de lecture ouvert contient une, deux, trois mutations silencieuses ou plus dans les signaux d'encapsidation du virus trouvés dans les nucléotides proximaux 3' et 5' dans le cadre de lecture ouvert ;
(iv) des signaux d'encapsidation trouvés dans la région codante proximale 5' du premier segment de gène de virus de la grippe ; et
(v) des signaux d'encapsidation trouvés dans la région non codante 5' du premier segment de gène de virus de la grippe ; et

(b) un deuxième segment de gène de virus de la grippe chimérique comprend, dans l'ordre présenté :

(i) des signaux d'encapsidation trouvés dans la région non codante 3' du deuxième segment de gène de virus de la grippe ;
(ii) des signaux d'encapsidation trouvés dans la région codante proximale 3' du deuxième segment de gène de virus de la grippe, dans lequel la région codante proximale 3' du deuxième segment de gène de virus de la grippe a été mutée pour éliminer tout codon d'initiation et empêcher la traduction de la séquence de la région codante proximale 3' ;
(iii) un cadre de lecture ouvert du premier segment de gène de virus de la grippe, dans lequel le cadre de lecture ouvert contient une, deux, trois mutations silencieuses ou plus dans les signaux d'encapsidation du virus trouvés dans les nucléotides proximaux 3' et 5' dans le cadre de lecture ouvert ;
(iv) des signaux d'encapsidation trouvés dans la région codante proximale 5' du deuxième segment de gène de virus de la grippe ; et
(v) des signaux d'encapsidation trouvés dans la région non codante 5' du deuxième segment de gène de virus de la grippe ;
dans lequel les premier et deuxième segments de gène sont deux segments de gène différents, et
dans lequel le virus recombinant de la grippe présente une aptitude réduite à se réassortir avec un virus de la grippe différent, de telle manière que le virus de la grippe descendant comportant les segments de gène combinés présente une compétence de réplication réduite, dans lequel un virus avec la compétence de réplication réduite est un virus qui produit des titres au moins 1 log, 1,5 logs, 2 logs, 25 logs, 3 logs, 3,5 logs, 4 logs, 4,5 logs, 5 logs, 5,5 logs, 6 logs, 6,5 logs, 7 logs, 7,5 logs, 8 logs, 8,5 logs, 9 logs ou 10 logs inférieurs chez la descendance réplicative que chez la descendance réplicative produite par un virus de la grippe de type sauvage du même type.

2. Virus de la grippe A, B ou C recombinant, comprenant des segments de gène de virus de la grippe, dans lequel au moins trois des segments de gène de virus de la grippe sont des segments de gène de virus de la grippe chimériques, dans lequel :

(a) un premier segment de gène de virus de la grippe chimérique comprend, dans l'ordre présenté :

(i) des signaux d'encapsidation trouvés dans la région non codante 3' d'un troisième segment de gène de virus de la grippe ;
(ii) des signaux d'encapsidation trouvés dans la région codante proximale 3' du troisième segment de gène de virus de la grippe, dans lequel la région codante proximale 3' du troisième segment de gène de virus de la grippe a été mutée pour éliminer tout codon un titre viral et empêcher la traduction de la séquence de la région codante proximale 3' ;

(iii) un cadre de lecture ouvert d'un premier segment de gène de virus de la grippe, dans lequel le cadre de lecture ouvert contient une, deux, trois mutations silencieuses ou plus dans les signaux d'encapsidation du virus trouvés dans les nucléotides proximaux 3' et 5' dans le cadre de lecture ouvert ;
(iv) des signaux d'encapsidation trouvés dans la région codante proximale 5' du troisième segment de gène de virus de la grippe ; et
(v) des signaux d'encapsidation trouvés dans la région non codante 5' du troisième segment de gène de virus de la grippe ; et

(b) un deuxième segment de gène de virus de la grippe chimérique comprend, dans l'ordre présenté :

(i) des signaux d'encapsidation trouvés dans la région non codante 3' du premier segment de gène de virus de la grippe ;
(ii) des signaux d'encapsidation trouvés dans la région codante proximale 3' du premier segment de gène de virus de la grippe, dans lequel la région codante proximale 3' du premier segment de gène de virus de la grippe a été mutée pour éliminer tout codon un titre viral et empêcher la traduction de la séquence de la région codante proximale 3' ;
(iii) un cadre de lecture ouvert d'un deuxième segment de gène de virus de la grippe, dans lequel le cadre de lecture ouvert contient une, deux, trois mutations silencieuses ou plus dans les signaux d'encapsidation du virus trouvés dans les nucléotides proximaux 3' et 5' dans le cadre de lecture ouvert ;
(iv) des signaux d'encapsidation trouvés dans la région codante proximale 5' du premier segment de gène de virus de la grippe ; et
(v) des signaux d'encapsidation trouvés dans la région non codante 5' du premier segment de gène de virus de la grippe ; et

(c) un troisième segment de gène de virus de la grippe chimérique comprenant, dans l'ordre présenté :

(i) des signaux d'encapsidation trouvés dans la région non codante 3' du deuxième segment de gène de virus de la grippe ;
(ii) des signaux d'encapsidation trouvés dans la région codante proximale 3' du deuxième segment de gène de virus de la grippe, dans lequel la région codante proximale 3' du deuxième segment de gène de virus de la grippe a été mutée pour éliminer tout codon un titre viral et empêcher la traduction de la séquence de la région codante proximale 3' ;
(iii) un cadre de lecture ouvert du troisième segment de gène de virus de la grippe, dans lequel le cadre de lecture ouvert contient une, deux, trois mutations silencieuses ou plus dans les signaux d'encapsidation du virus trouvés dans les nucléotides proximaux 3' et 5' dans le cadre de lecture ouvert ;
(iv) des signaux d'encapsidation trouvés dans la région codante proximale 5' du deuxième segment de gène de virus de la grippe ; et
(v) des signaux d'encapsidation trouvés dans la région non codante 5' du deuxième segment de gène de virus de la grippe ;
dans lequel les premier, deuxième et troisième segments de gène sont trois segments de gène différents, et dans lequel le virus recombinant de la grippe présente une aptitude réduite à se réassortir avec un virus de la grippe différent, de telle manière que le virus de la grippe descendant comportant les segments de gène combinés présente une compétence de réplication réduite, dans lequel un virus avec la compétence de réplication réduite est un virus qui produit des titres au moins 1 log, 1,5 logs, 2 logs, 25 logs, 3 logs, 3,5 logs, 4 logs, 4,5 logs, 5 logs, 5,5 logs, 6 logs, 6,5 logs, 7 logs, 7,5 logs, 8 logs, 8,5 logs, 9 logs ou 10 logs inférieurs chez la descendance réplicative que chez la descendance réplicative produite par un virus de la grippe de type sauvage du même type.

3. Virus de la grippe recombinant selon la revendication 1 ou 2, dans lequel le premier, deuxième ou troisième segment de gène de virus de la grippe fait référence à un segment de gène HA, NA, NS, PB1, PB2, PA, M, ou NP d'un virus de la grippe.

4. Virus de la grippe recombinant selon la revendication 1, dans lequel le premier segment de gène de virus de la grippe chimérique code pour une protéine HA et le deuxième segment de gène de virus de la grippe chimérique code pour une protéine NA.

5. Virus de la grippe recombinant selon la revendication 4, dans lequel le deuxième segment de gène de virus de la grippe chimérique comprend :

(a) la séquence nucléotidique de la région non codante 3' constituée de la SEQ ID N° : 19 et la séquence de la région codante proximale 3' constituée de la SEQ ID N° : 20, et
(b) la séquence nucléotidique de la région non codante 5' constituée de la SEQ ID N° : 22 et la séquence de la région codante proximale 5' constituée de la SEQ ID N° : 23.

6. Virus de la grippe recombinant selon la revendication 4 ou 5, dans lequel le premier segment de gène de virus de la grippe chimérique comprend :

(a) la séquence nucléotidique de la région non codante 3' constituée de la SEQ ID N° : 31 et la séquence de la région codante proximale 3' constituée de la SEQ ID N° : 32, et
(b) la séquence nucléotidique de la région non codante 5' constituée de la SEQ ID N° : 34 et une séquence de la région codante proximale 5' constituée de la SEQ ID N° : 35.

7. Virus de la grippe recombinant selon la revendication 2, dans lequel les premier, deuxième et troisième segments de gène de virus de la grippe codent pour les protéines HA, NA et NS, respectivement.

8. Virus recombinant de la grippe selon la revendication 1, dans lequel

(a) le premier segment de gène de virus de la grippe chimérique code pour une protéine NS, et dans lequel la région codante proximale 3' est mutée de façon à éliminer le site d'épissage 5' de l'ARNm ; ou
(b) le premier segment de gène de virus de la grippe chimérique code pour une protéine M, et dans lequel la région codante proximale 3' est mutée de façon à éliminer le site d'épissage 5' de l'ARNm.

9. Virus de la grippe recombinant selon l'une quelconque des revendications 1 à 8, dans lequel le virus de la grippe recombinant se réassortit avec d'autres virus de la grippe par moins de 5 %, tel que déterminé par le pourcentage de plaques virales contenant les virus de la grippe réassortis avec un ou plusieurs segments de gène de virus de la grippe chimériques qui se sont réassortis indépendamment d'un ou plusieurs autres segments de gène de virus de la grippe chimériques.

10. Virus de la grippe recombinant selon l'une quelconque des revendications 1 à 9, dans lequel le virus est atténué.

11. Hôte comprenant le virus de la grippe recombinant selon l'une quelconque des revendications 1 à 10, dans lequel l'hôte est une cellule de poulet ou aviaire, une cellule d'une ligne cellulaire, ou un embryophore de poulet ou aviaire.

12. Composition pharmaceutique ou composition immunogène comprenant le virus de la grippe recombinant selon l'une quelconque des revendications 1 à 10.

13. Virus de la grippe recombinant selon l'une quelconque des revendications 1 à 10, ou composition selon la revendication 12, pour une utilisation dans la provocation d'une réponse immunitaire contre un virus de la grippe chez un sujet.

14. Virus de la grippe recombinant selon l'une quelconque des revendications 1 à 10, ou composition selon la revendication 12, pour une utilisation dans la prévention ou le traitement d'une maladie du virus de la grippe chez un sujet.

15. Virus de la grippe recombinant selon l'une quelconque des revendications 1 à 10, ou composition selon la revendication 12, pour une utilisation dans le traitement d'une infection par le virus de la grippe chez un sujet.

16. Virus de la grippe recombinant pour une utilisation selon l'une quelconque des revendications 13 à 15, dans lequel le sujet est un être humain.

17. Procédé *in vitro* de propagation d'un virus de la grippe recombinant, dans lequel le procédé comprend l'infection d'une cellule de poulet ou aviaire ou d'une cellule d'une lignée cellulaire par le virus de la grippe recombinant selon l'une quelconque des revendications 1 à 10 ; et ensuite la purification du de ladite cellule.

18. Procédé *in ovo* de propagation d'un virus de la grippe recombinant, dans lequel le procédé comprend l'infection d'un embryophore de poulet ou aviaire par le virus de la grippe recombinant selon l'une quelconque des revendications 1 à 10 ; et ensuite la purification du virus à partir dudit oeuf.

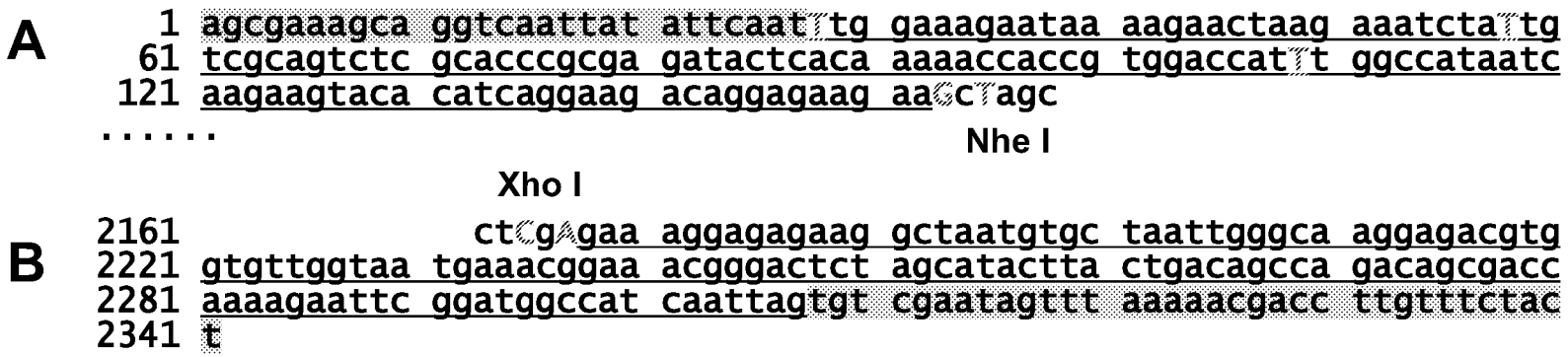

Fig. 1A-1B

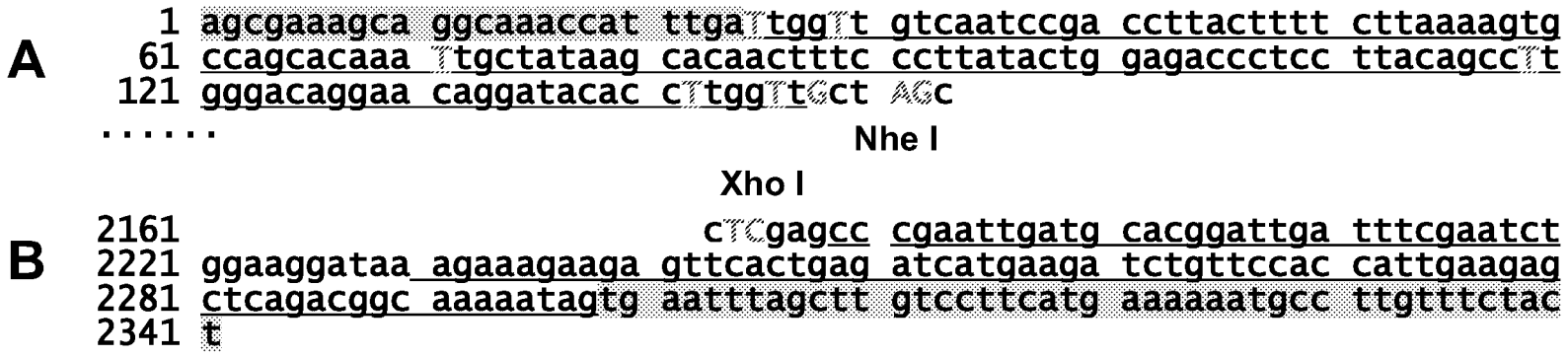

Fig. 2A-2B

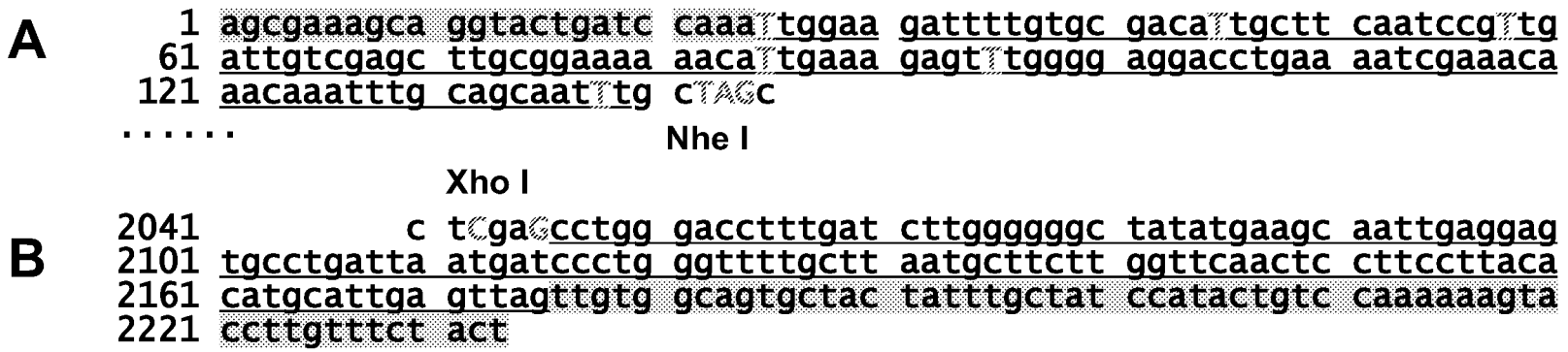

Fig. 3A-3B

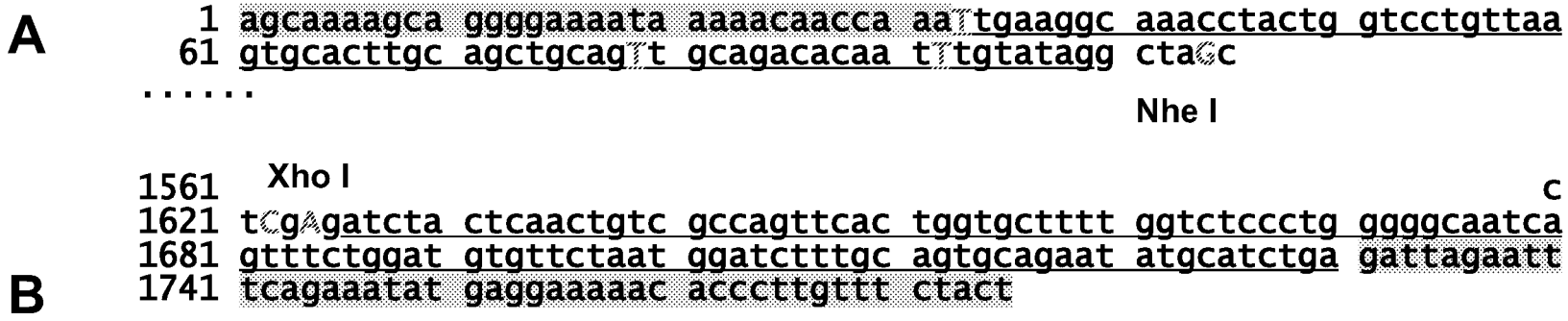

Fig. 4A-4B

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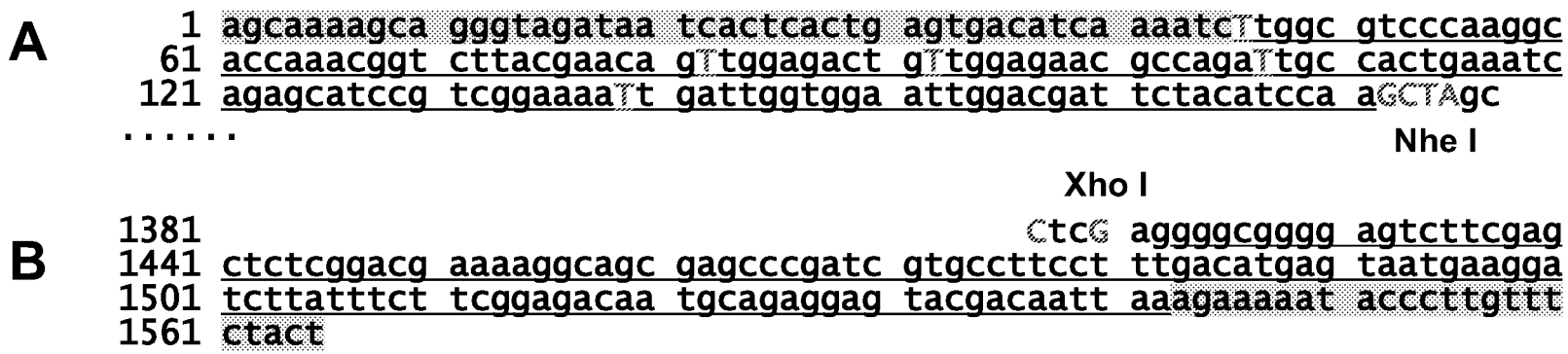


Fig. 5A-5B

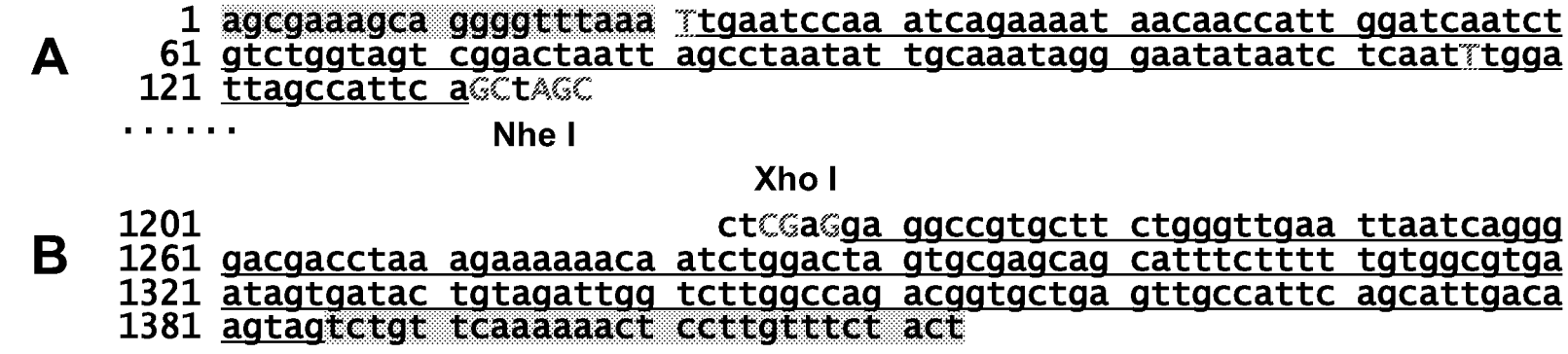


Fig. 6A-6B

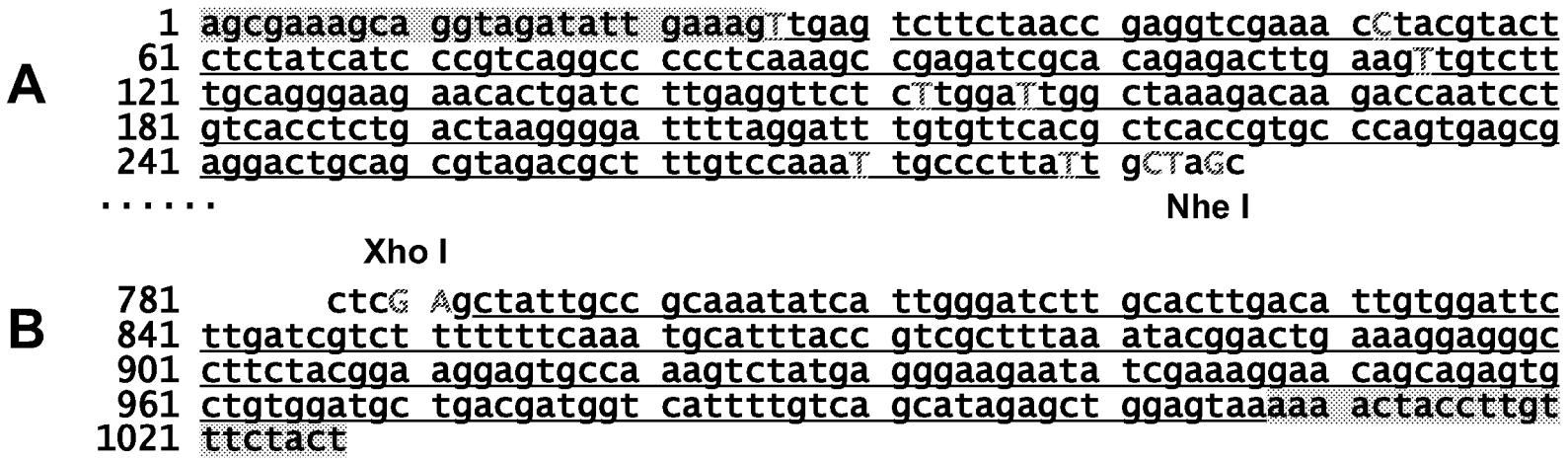


Fig. 7A-7B

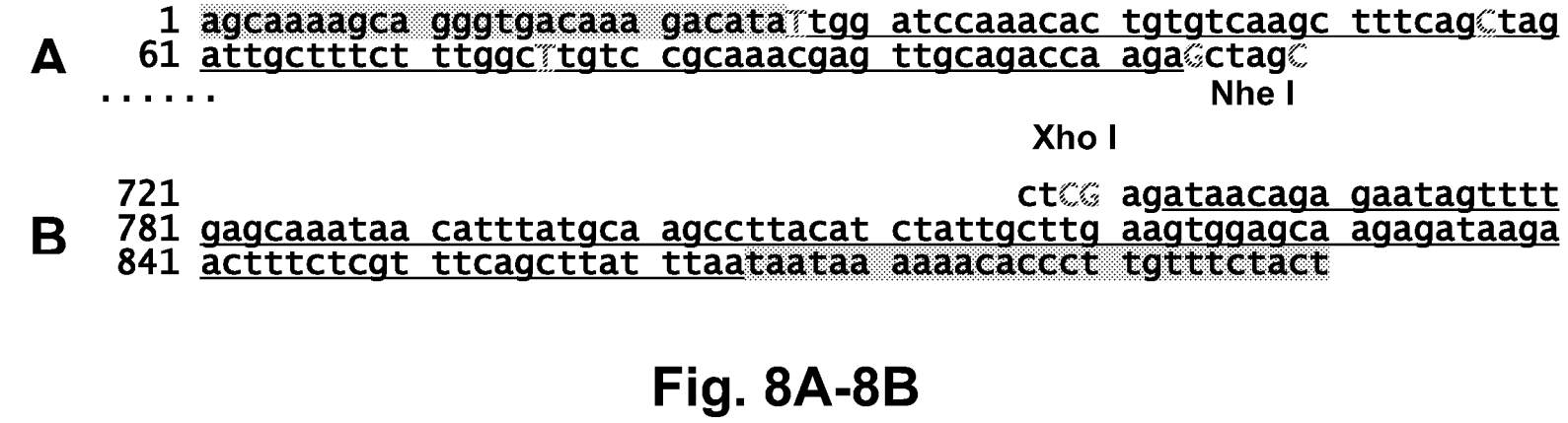


Fig. 8A-8B

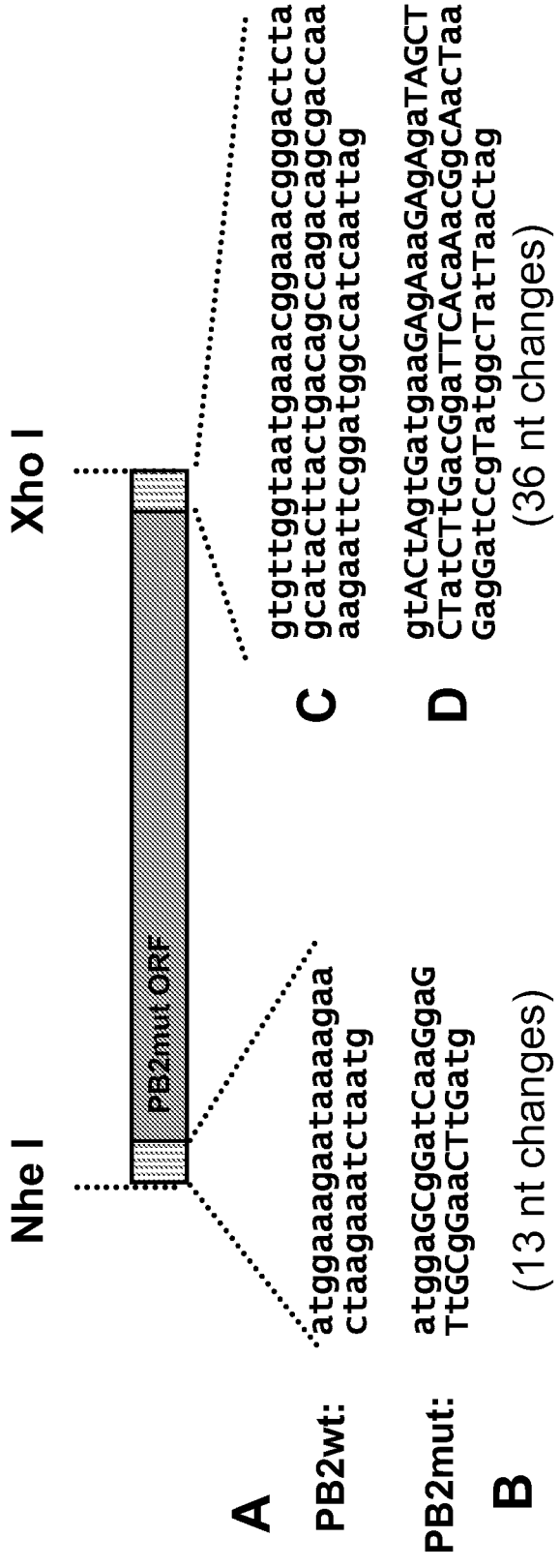

Nhe I
Xho I
PB2mut ORF
A
PB2wt:
atggaaagaataaaagaa
ctaagaaatctaatg
PB2mut:
atggaGCgGatCaaGgaG
TtGCgGaaCTtGatg
B
(13 nt changes)
C
gtgttggtaatgaaacggaaacgggactcta
gcatacttactgacagccagacagcgaccaa
aagaattcggatggccatcaattag
D
gtACtAgtGatgaaGAgAaaGAgAgaTAGCT
CTatCTtGacGgaTTCAcaAacGgcAacTaa
GagGatCcgTatggcTatTaaCtag
(36 nt changes)

Fig. 9A-9D

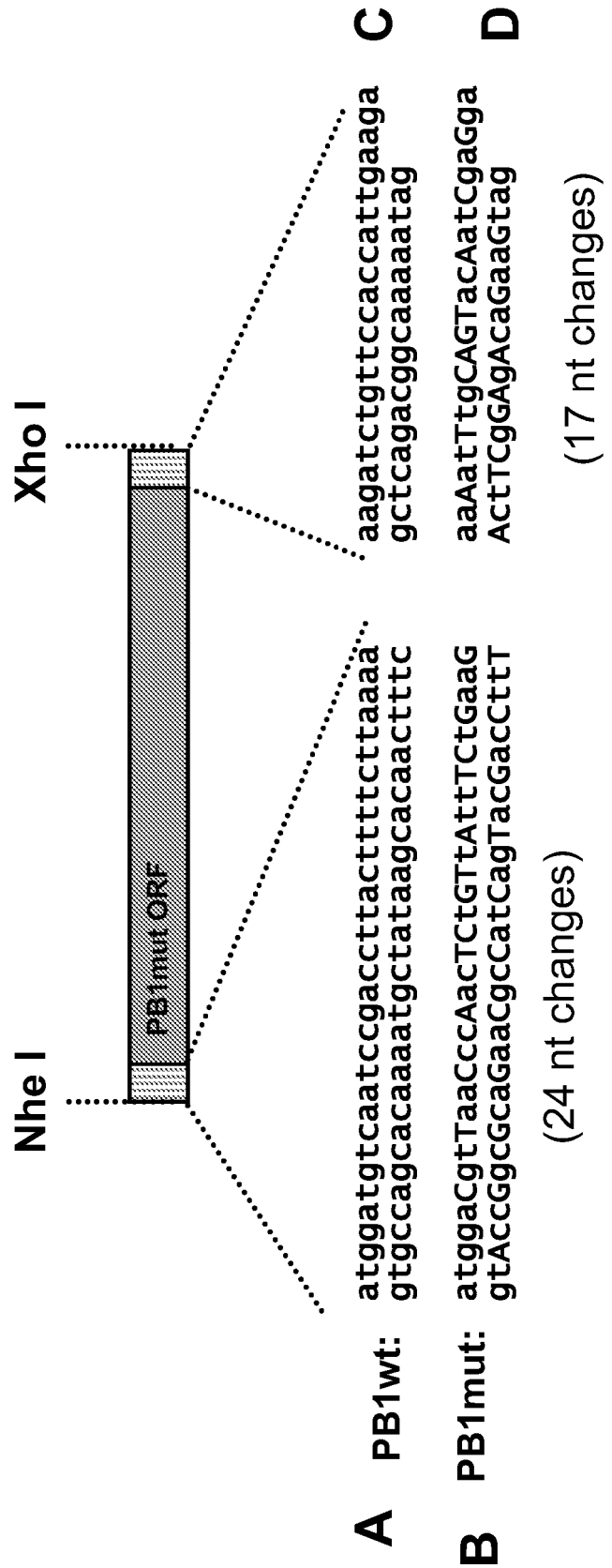

Nhe I
Xho I
PB1mut ORF
A
PB1wt:
atggatgtcaatccgaccttactttttcttaaaa
gtgccagcacaaaaatgctataagcacaactttc
B
PB1mut:
atggaCgtTaaCccAacTCtGTtAttTCtGaaG
gtAccGgcGcaGaaCgcCatCagTacGacCttT
(24 nt changes)
C
aagatctgttccaccattgaaga
gctcagacggcaaaaatag
D
aaAatTtgCAGTacAatCgaGga
ActTCgGAgAcaGaaGtag
(17 nt changes)

Fig. 10A-10D

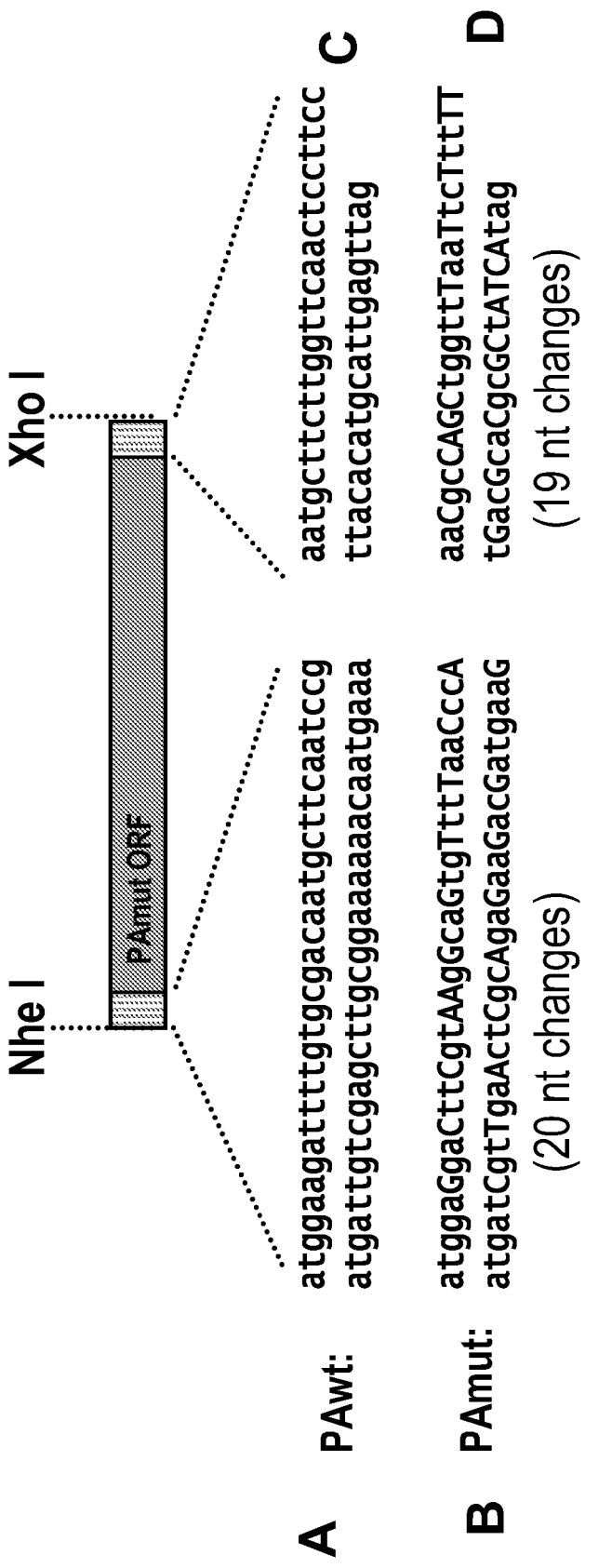

Nhe I
Xho I
PAmut ORF
A
PAwt:
atggaagattttgtgcgacaatgcttcaatccg
atgattgtcgagcttgcggaaaaaacaatgaaa
B
PAmut:
atggaGgaCttCgtAAgGcaGtgTtttTaaCccA
atgatCgtTgaActCgCAgaGaaGacGatgaaG
(20 nt changes)
C
aatgcttcttggttcaactccttcc
ttacacatgcattgagttag
D
aaCgcCAGCtggtttTaaTtcTtttT
tGacGcaCgcGCtATCAtag
(19 nt changes)

Fig. 11A-11D

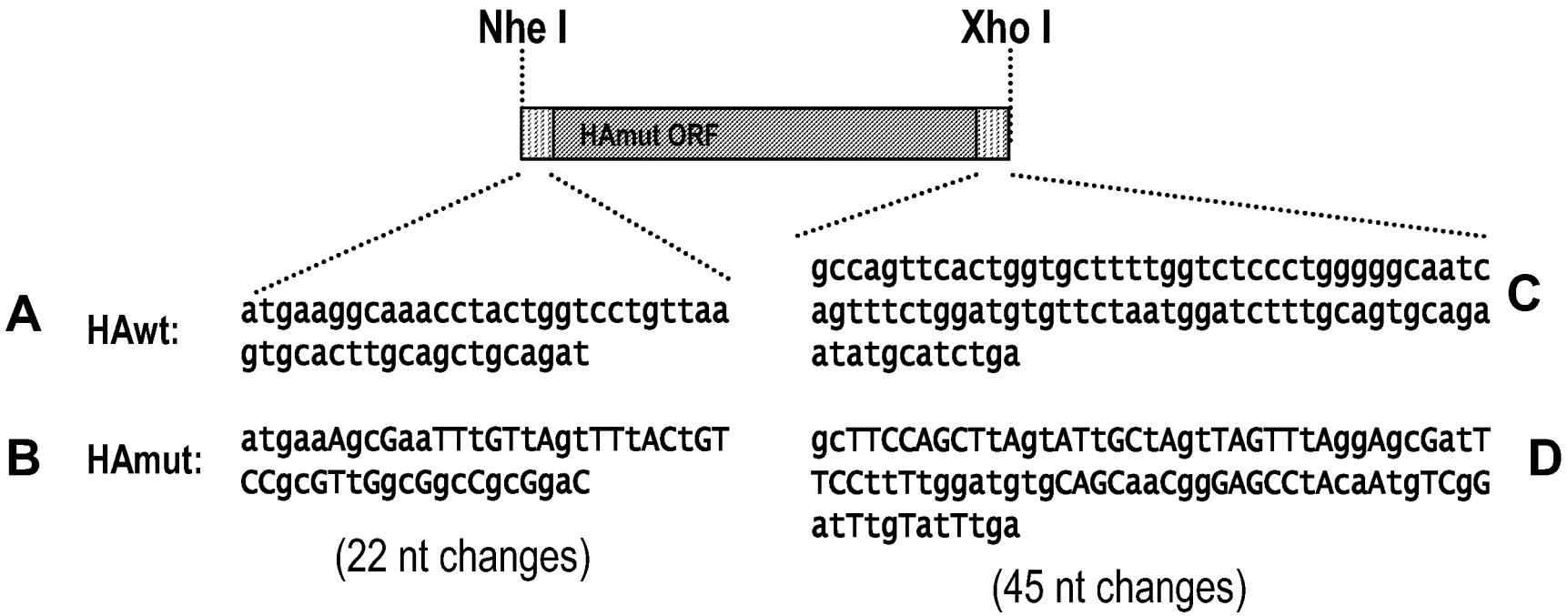

Nhe I
Xho I
HAmut ORF
A
HAwt:
atgaaggcaaacctactggtcctgttaa
gtgcacttgcagctgcagat
gccagttcactggtgcttttggtctccctgggggcaatc
agtttctggatgtgttctaatggatctttgcagtgcaga
atatgcatctga
C
B
HAmut:
atgaaAgcGaaTTtGTtAgtTTtACtGT
CCgcGTtGgcGgcCgcGgaC
(22 nt changes)
gcTTCCAGCTtAgtATtGCtAgtTAGTTtAggAgcGatT
TCCttTtggatgtgCAGCaaCggGAGCCtAcaAtgTCgG
atTtgTatTtga
(45 nt changes)
D

Fig. 12A-12D

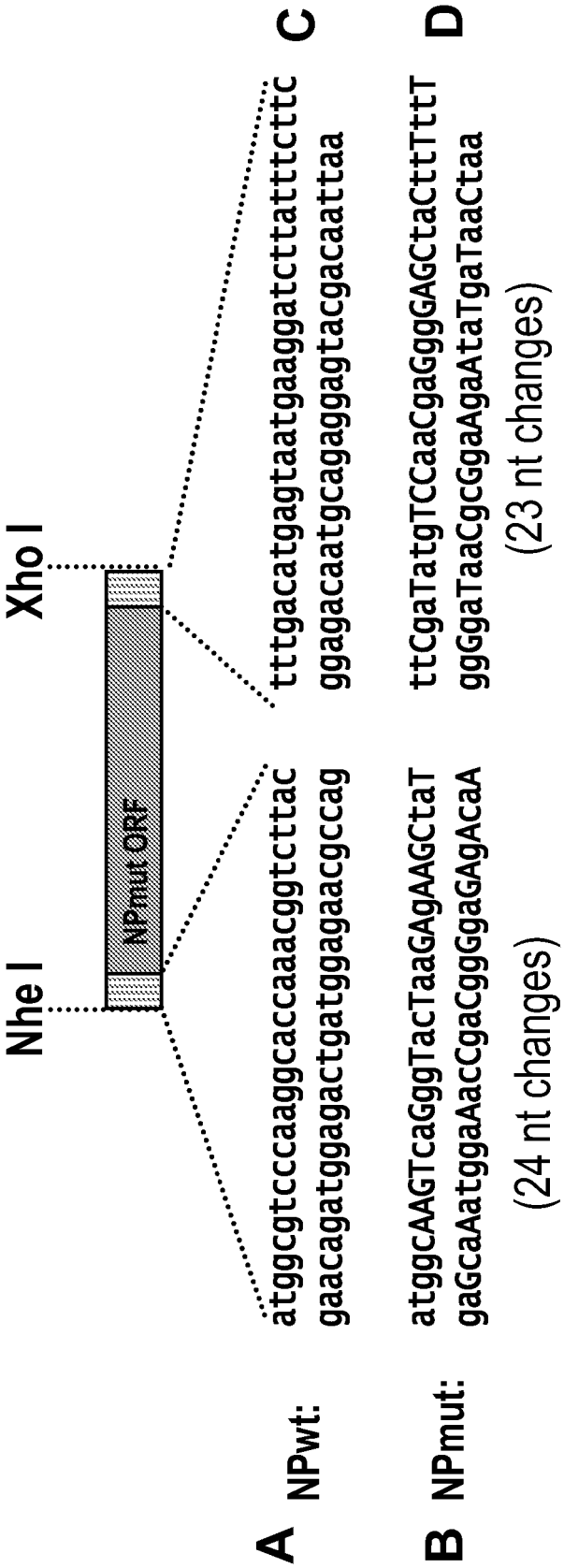

Nhe I
Xho I
NPmut ORF
A NPwt:
atggcgtcccaaggcaccaaacggtcttac
gaacagatggagactgatggagaacgccag
B NPmut:
atggcAAGTcaGggTacTaaGAgAAGCtaT
gaGcaAatggaAacCgaCggGgaGAgAcaA
(24 nt changes)
C
tttgacatgagtaatgaaggatcttatttcttc
ggagacaatgcagaggagtacgacaattaa
D
ttCgaTatgTCCaaCgaGggGAGCtaCttTttT
ggGgaTaaCgcGgaAgaAtaTgaTaaCtaa
(23 nt changes)

Fig. 13A-13D

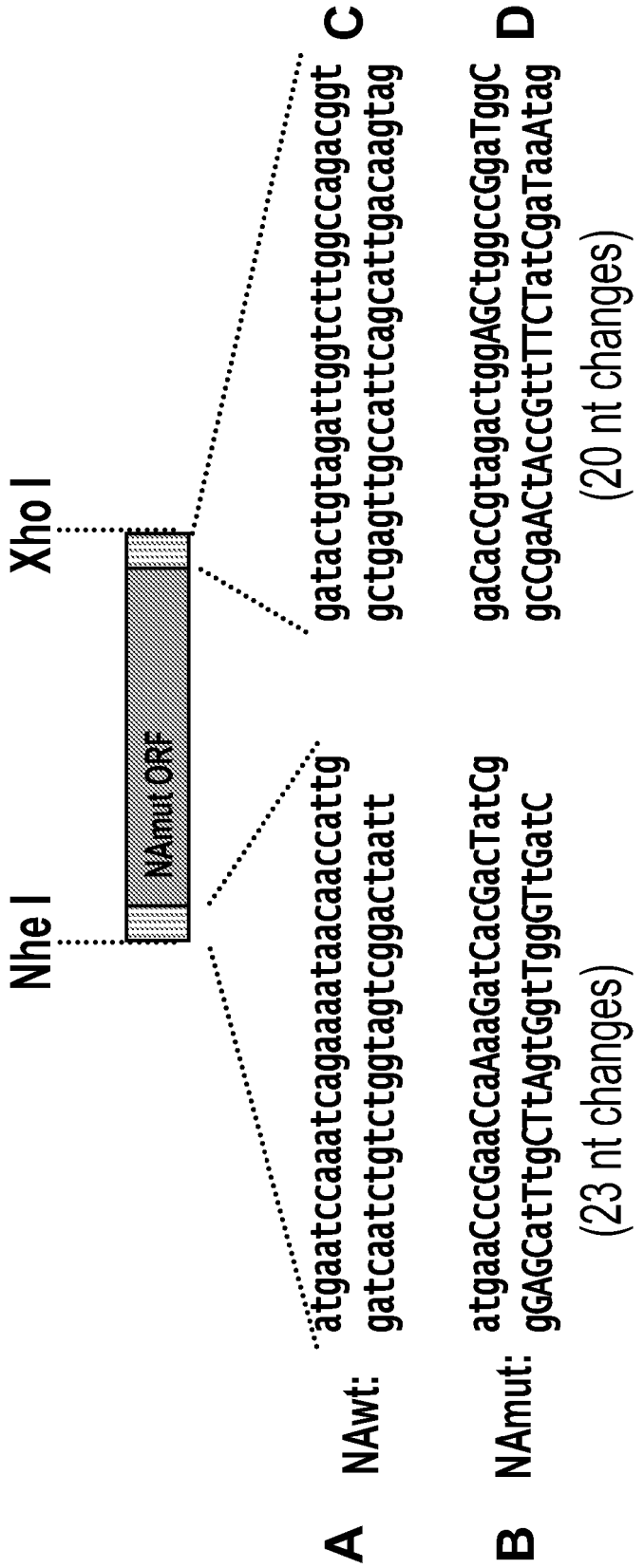

Nhe I
Xho I
NAmut ORF
A
NAwt:
atgaatccaaatcagaaaataacaaccattg
gatcaatctgtctggtagtcggactaatt
B
NAmut:
atgaaCccGaaCcaAaaGatCacGacTatCg
gGAGCatTtgCTtAgtGgtTggGTtGatC
(23 nt changes)
C
gatactgtagattggtcttggccagacggt
gctgagttgccattcagcattgacaagtag
D
gaCacCgtagactggAGCtggccGgaTggC
gcCgaACtAccGttTTCTatCgaTaaAtag
(20 nt changes)

Fig. 14A-14D

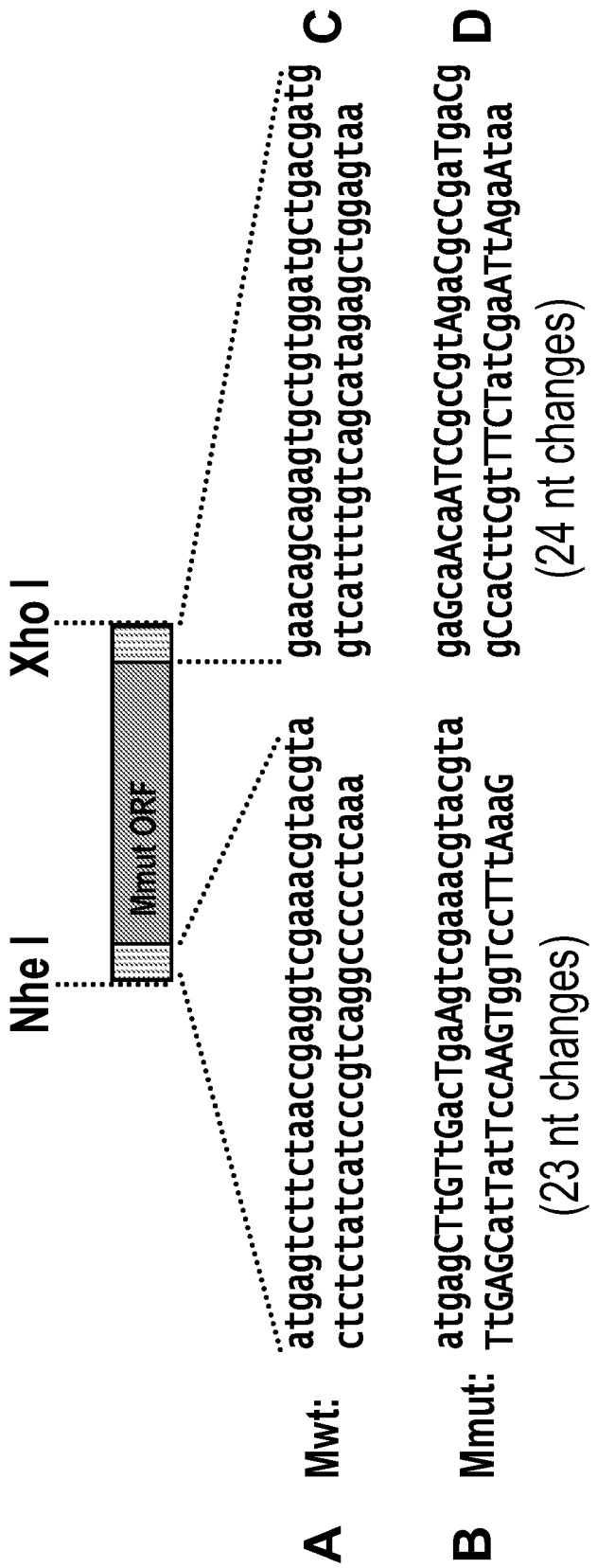

Nhe I
Xho I
Mmut ORF
A
Mwt:
atgagtcttctaaccgaggtcgaaacgtacgta
ctctctatcatcccgtcaggcccccctcaaa
B
Mmut:
atgagCTtGTtGacTgaAgtcgaaacgtacgta
TtGAGCatTatTccAAGTggTccTTtAaaG
(23 nt changes)
C
gaacagcagagtgctgtggatgctgacgatg
gtcattttgtcagcatagagctggagtaa
D
gaGcaAcaATCCgcCgtAgaCgcCgaTgaCg
gCcaCttCgtTTCTatCgaATtAgaAtaa
(24 nt changes)

Fig. 15A-15D

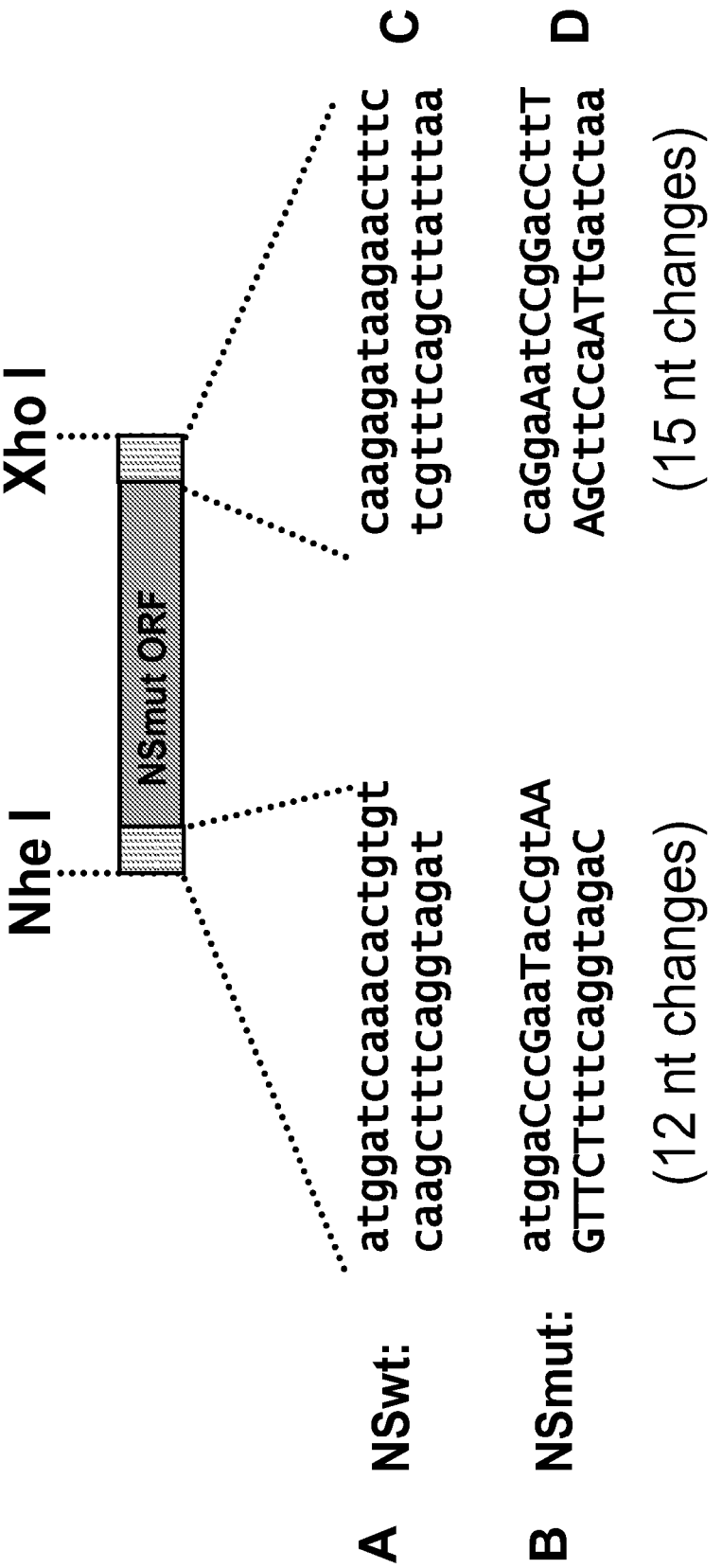

Xho I
Nhe I
NSmut ORF
A
NSwt:
atggatccaaacactgtgt
caagctttcaggtagat
B
NSmut:
atggaCccGaaTaccgtAA
GTTCTtttcaggtagaC
(12 nt changes)
C
caagagataagaactttc
tcgtttcagcttatttaa
D
caGgaAatCCgGaccttT
AGCttCcaATtGatCtaa
(15 nt changes)

Fig. 16A-16D

```
   1 agcaaaagca ggggaaaata aaaacaacca aaatgaaggc aaaactactg gtcctgttat
  61 atgcatttgt agctacagat gcagacacaa tatgtatagg ctaccatgcg aacaactcaa
 121 ccgacactgt tgacacaata ttcgagaaga atgtggcagt gacacattct gttaacctgc
 181 tcgaagacag acacaacggg aaactatgta aattaaaagg aatagcccca ctacaattgg
 241 ggaaatgtaa catcaccgga tggctcttgg gaaatccaga atgcgactca ctgcttccag
 301 cgagatcatg gtcctacatt gtagaaacac caaactctga gaatggagca tgttatccag
 361 gagatttcat cgactatgag gaactgaggg agcaattgag ctcagtatca tcattagaaa
 421 gattcgaaat atttcccaag gaaagttcat ggcccaacca cacattcaac ggagtaacag
 481 tatcatgctc ccatagggga aaaagcagtt tttacagaaa tttgctatgg ctgacgaaga
 541 agggggattc atacccaaag ctgaccaatt cctatgtgaa caataaaggg aaagaagtcc
 601 ttgtactatg gggtgttcat cacccgtcca gcagtgatga gcaacagagt ctctatagta
 661 atggaaatgc ttatgtctct gtagcgtctt caaattataa caggagattc accccggaaa
 721 tagctgcaag gcccaaagta aaagatcaac atgggaggat gaactattac tggaccttgc
 781 tagaacccgg agacacaata atatttgagg caactggtaa tctaatagca ccatggtatg
 841 ctttcgcact gagtagaggg tttgagtccg gcatcatcac ctcaaacgcg tcaatgcatg
 901 agtgtaacac gaagtgtcaa acaccccagg gatctataaa cagcaatctc cctttccaga
 961 atatacaccc agtcacaata ggagagtgcc caaaatatgt caggagtacc aaattgagga
1021 tggttacagg actaagaaac atcccatcca ttcaatacag aggtctattt ggagccattg
1081 ctggttttat tgaggggga tggactggaa tgatagatgg atggtatggt tatcatcatc
1141 agaatgaaca gggatcaggc tatgcagcgg atcaaaaaag cacacagaat gccattaacg
1201 ggattacaaa caaggtgaac tctgttatcg agaaaatgaa cactcaattc acagctgtgg
1261 gtaaagaatt caacaactta gaaaaaagga tggaaaattt aaataaaaaa gttgatgatg
1321 ggtttctgga catttggaca tataatgcag aattgttagt tctactggaa aatgaaagaa
1381 ctttggattt ccatgactta aatgtgaaga atctgtacga gaaagtaaaa agccaattaa
1441 agaataatgc caaagaaatc ggaaatgggt gttttgagtt ctaccacaag tgtgacaatg
1501 aatgcatgga aagtgtaaga aatgggactt atgattatcc aaaatattca gaagaatcaa
1561 agttgaacag ggaaaagata gatggagtga aattggaatc aatgggggtg tatcagattc
1621 tggcgatcta ctcaactgtc gccagttcac tggtgctttt ggtctccctg ggggcaatca
1681 gtttctggat gtgttctaat gggtctttgc agtgcagaat atgcatctga gattaggatt
1741 tcagaaatat aaggaaaaac acccttgttt ctact
```

FIG. 17

```
   1 agcgaaagca ggagtttaaa tgaatccaaa ccagaaaata ataaccattg ggtcaatctg
  61 tatggtagtc ggaataatta gcctaatatt gcaaatagga aatataatct caatatggat
 121 tagccattca attcaaaccg gaaatcaaaa ccatactgga atatgcaacc aaggcagcat
 181 tacctataaa gttgttgctg ggcaggactc aacttcagtg atattaaccg gcaattcatc
 241 tctttgtccc atccgtgggt gggctataca cagcaaagac aatggcataa gaattggttc
 301 caaaggagac gtttttgtca taagagagcc ttttatttca tgttctcact tggaatgcag
 361 gacctttttt ctgactcaag gcgccttact gaatgacaag cattcaaggg ggacctttaa
 421 ggacagaagc ccttataggg ccttaatgag ctgccctgtc ggtgaagctc cgtccccgta
 481 caattcaagg tttgaatcgg ttgcttggtc agcaagtgca tgtcatgatg gagtgggctg
 541 gctaacaatc ggaatttctg gtccagatga tggagcagtg gctgtattaa aatacaaccg
 601 cataataact gaaaccataa aaagttggag gaagaatata ttgagaacac aagagtctga
 661 atgtacctgt gtaaatggtt catgttttac cataatgacc gatggcccaa gtgatgggct
 721 ggcctcgtac aaaattttca agatcgagaa ggggaaggtt actaaatcaa tagagttgaa
 781 tgcacctaat tctcactacg aggaatgttc ctgttaccct gataccggca aagtgatgtg
 841 tgtgtgcaga gacaattggc acggttcgaa ccgaccatgg gtgtccttcg accaaaacct
 901 agattataaa ataggataca tctgcagtgg ggttttcggt gacaacccgc gtcccaaaga
 961 tggaacaggc agctgtggcc cagtgtctgc tgatggagca aacggagtaa agggattttc
1021 atataagtat ggcaatggtg tttggatagg aaggactaaa agtgacagtt ccagacatgg
1081 gtttgagatg atttgggatc ctaatggatg gacagagact gatagtaggt tctctatgag
1141 acaagatgtt gtggcaataa ctaatcggtc agggtacagc ggaagtttcg ttcaacatcc
1201 tgagctaaca gggctagact gtatgaggcc ttgcttctgg gttgaattaa tcaggggct
1261 acctgaggag gacgcaatct ggactagtgg gagcatcatt tctttttgtg gtgtgaatag
1321 tgatactgta gattggtctt ggccagacgg tgctgagttg ccgttcacca ttgacaagta
1381 gtttgttcaa aaaactcctt gtttctact
```

FIG. 18

```
   1 agcaaaagca ggtagatatt gaaagatgag tcttctaacc gaggtcgaaa cgtacgttct
  61 ctctatcgtc ccgtcaggcc ccctcaaagc cgagatcgca cagagacttg aagatgtctt
 121 tgcagggaag aacaccgatc ttgaggttct catggaatgg ctaaagacaa gaccaatcct
 181 gtcacctctg actaagggga ttttaggatt tgtgttcacg ctcaccgtgc ccagtgagcg
 241 gggactgcag cgtagacgct ttgtccaaaa tgctcttaat gggaacggag atccaaataa
 301 catggacaaa gcagttaaac tgtataggaa gcttaagagg gagataacat tccatggggc
 361 caaagaaata gcactcagtt attctgctgg tgcacttgcc agttgtatgg gcctcatata
 421 caacaggatg ggggctgtgg ccactgaagt ggcatttggc ctggtatgcg caacctgtga
 481 acagattgct gactcccagc atcggtctca taggcaaatg gtgacaacaa ccaatccact
 541 aatcagacat gagaacagaa tggttctagc cagcactaca gctaaggcta tggagcaaat
 601 ggctggatcg agtgagcaag cagcagaggc catggatatt gctagtcagg ccaggcaaat
 661 ggtgcaggcg atgagaacca ttgggactca tcctagctcc agtactggtc taaaagatga
 721 tcttcttgaa aatttgcagg cctatcagaa acgaatgggg gtgcagatgc aacgattcaa
 781 gtgatcctct cgttattgca gcaaatatca ttgggatctt gcacttgata ttgtggattc
 841 ttgatcgtct ttttttcaaa tgcatttatc gtcgctttaa atacggtttg aaaagagggc
 901 cttctacgga aggagtgcca gagtctatga gggaagaata tcgaaaggaa cagcagaatg
 961 ctgtggatgt tgacgatggt cattttgtca acatagagct ggagtaaaaa actaccttgt
1021 ttctact
```

FIG. 19

```
  1 agcaaaagca gggtgacaaa gacataatgg atccaaacac tgtgtcaagc tttcaggtag
 61 attgctttct ttggcatgtc cgcaaaagag ttgcagacca agaactaggt gattccccat
121 tccttgatcg gcttcgccga gatcagaagt ccctaagagg aagaggcagc actctcggtc
181 tggacatcga aacagccacc cgtgctggaa agcaaatagt ggagcggatt ctgaaggaag
241 aatccgatga ggcacttaaa atgaccatgg cctctgtacc tgcatcgcgc tacctaactg
301 acatgactct tgaggaaatg tcaaggcact ggttcatgct catgcccaag cagaaagtgg
361 caggccctct ttgtatcaga atggaccagg cgatcatgga taagaacatc atactgaaag
421 cgaacttcag tgtgattttt gaccggctgg agactctaat attactaagg gccttcaccg
481 aagagggaac aattgttggc gaaatttcac cactgccttc tcttccagga catactgatg
541 aggatgtcaa aaatgcagtt ggggtcctca tcggaggact tgaatggaat aataacacag
601 ttcgagtctc tgaaactcta cagagattcg cttggagaag cagtaatgag aatgggagac
661 ctccactcac tccaaaacag aaacgaaaaa tggcgggaac aattaggtca gaagtttgaa
721 gaaataagat ggttgattga agaagtgaga cacagactga agataacaga gaatagtttt
781 gagcaaataa catttatgca agccttacaa ctattgcttg aagtggagca agagataaga
841 actttctcgt ttcagcttat ttaataataa aaaacaccct tgtttctact
```

FIG. 20

```
   1 agcgaaagca ggtactgatt caaaatggaa gattttgtgc gacaatgctt caatccgatg
  61 attgtcgagc ttgcggaaaa ggcaatgaaa gagtatggag aggacctgaa aatcgaaaca
 121 aacaaatttg cagcaatatg cactcacttg gaagtgtgct tcatgtattc agattttcac
 181 ttcatcgatg agcaaggcga gtcaatagtc gtagaacttg gcgatccaaa tgcacttttg
 241 aagcacagat ttgaaataat cgagggaaga gatcgcacaa tagcctggac agtaataaac
 301 agtatttgca acactacagg ggctgagaaa ccaaagtttc taccagattt gtatgattac
 361 aagaagaata gattcatcga aattggagta acaaggagag aagttcacat atactatctg
 421 gaaaaggcca ataaaattaa atctgagaag acacacatcc acattttctc attcactggg
 481 gaggaaatgg ccacaaaggc cgactacact ctcgatgaag aaagcagggc taggatcaaa
 541 accaggctat tcaccataag acaagaaatg gctagcagag gcctctggga ttcctttcgt
 601 cagtccgaga gaggcgaaga gacaattgaa gaaagatttg aaatcacagg aacaatgcgc
 661 aagcttgccg accaaagtct cccgccaaac ttctccagcc ttgaaaattt tagagcctat
 721 gtggatggat tcgaaccgaa cggctacatt gagggcaagc tttctcaaat gtccaaagaa
 781 gtaaatgcta gaattgaacc ttttttgaaa tcaacaccac gaccacttag acttccggat
 841 gggcctccct gttctcagcg gtccaaattc ctgctgatgg atgccttaaa attaagcatt
 901 gaggacccaa gtcatgaggg agaggggata ccgctatatg atgcaatcaa atgcatgaga
 961 acattctttg gatggaagga acccaatgtt gttaaaccac acgaaaaggg aataaatcca
1021 aattatcttc tgtcatggaa gcaagtactg gcagaactgc aggacattga gaatgaggag
1081 aaaattccaa ggactaaaaa tatgaagaaa acgagtcagt taaagtgggc acttggtgag
1141 aacatggcac cagaaaaggt agactttgac gattgtaaag atgtaggcga tttgaagcaa
1201 tatgatagtg atgaaccaga attgaggtcg cttgcaagtt ggattcagaa tgagttcaac
1261 aaggcatgtg aactgaccga ttcaagctgg atagagctcg atgagattgg agaagatgcg
1321 gctccaattg aacacattgc aagcatgaga aggaattatt tcacagcaga ggtgtctcat
1381 tgcagagcca cagaatacat aatgaagggg gtgtacatca atactgcctt gcttaatgca
1441 tcctgtgcag caatggatga tttccaatta attccaatga taagcaagtg tagaactaag
1501 gagggaaggc gaaagaccaa tttgtacggt ttcatcataa aaggaagatc ccacttaagg
1561 aatgacaccg atgtggtaaa ctttgtgagc atggagtttt ccctcactga cccaagactt
1621 gaaccacaca aatgggagaa gtactgtgtt cttgaggtag gagatatgct tctaagaagt
1681 gccataggcc atgtgtcaag gcctatgttc ttgtatgtga ggacaaatgg aacctcaaaa
1741 attaaaatga aatgggggat ggaaatgagg cgttgcctcc ttcagtcact tcaacaaatc
1801 gagagtatga ttgaagctga gtcctctgtc aaggagaaag acatgaccaa agagttcttt
1861 gaaaacaaat cagaaacatg gcccgttgga gagtccccca aaggagtgga ggaaggttcc
1921 attgggaagg tctgcagaac tttattggca aagtcggtat tcaacagctt gtatgcatct
1981 ccacaactgg aaggattttc agctgaatca agaaaactgc ttcttatcgt tcaggctctt
2041 agggacaacc tggaacctgg gacctttgat cttggggggc tatatgaagc aattgaggag
2101 tgcctgatta atgatccctg ggttttgctt aatgcttctt ggttcaactc cttcctcaca
2161 catgcattga gatagttgtg gcaatgctac tatttgctat ccatactgtc caaaaaagta
2221 ccttgtttct act
```

FIG. 21

```
   1 agcgaaagca ggcaaaccat ttgaatggat gtcaatccga ctttactttt cttaaaagtg
  61 ccagcacaaa atgctataag cacaactttc ccttatactg gagaccctcc ttacagccat
 121 gggacaggaa caggatacac catggatact gtcaacagga cacatcagta ctcagaaagg
 181 ggaagatgga caacaaacac cgaaactgga gcaccgcaac tcaacccgat tgatgggcca
 241 ctgccagaag acaatgaacc aagtggttat gcccaaacag attgtgtatt ggaagcaatg
 301 gccttccttg aggaatccca tcctggtatc tttgagacct cgtgtcttga aacgatggag
 361 gttgttcagc aaacacgagt ggacaagctg acacaaggcc gacagaccta tgactggact
 421 ctaaatagga accagcctgc tgcaacagca ttggccaaca caatagaagt gttcagatca
 481 aatggcctca cggccaatga atccggaagg ctcatagact tccttaagga tgtaatggag
 541 tcaatgaaca aagaagaaat ggagatcaca actcattttc agagaaagag acgagtgaga
 601 gacaatatga ctaagaaaat ggtgacacag agaacaatag gtaaaaggaa gcagagattg
 661 aacaaaagga gttatctaat tagggcattg accctgaaca caatgaccaa agatgctgag
 721 agagggaagc taaaacggag agcaattgca accccaggga tgcaaataag ggggtttgta
 781 tactttgttg agacactagc aaggagtata tgtgagaaac ttgaacaatc aggattgcca
 841 gttggaggca atgagaagaa agcaaagttg gcaaatgttg taaggaagat gatgaccaat
 901 tctcaggaca ctgaaatttc tttcaccatc actggagata acaccaaatg gaacgaaaat
 961 cagaaccctc ggatgttttt ggccatgatc acatatataa ccagaaatca gcccgaatgg
1021 ttcagaaatg ttctaagtat tgctccaata atgttctcaa acaaaatggc gagactggga
1081 aaggggtaca tgtttgagag caagagtatt aaaattagaa ctcaaatacc tgcagaaatg
1141 ctagcaagca tcgatttgaa atacttcaat gattcaacta gaaagaagat tgaaaaaatc
1201 cggccgctct taatagatgg gactgcatca ttgagccctg gaatgatgat gggcatgttc
1261 aatatgttaa gtactgtatt aggcgtctcc atcctgaatc ttggacaaaa gagacacacc
1321 aagactactt actggtggga tggtcttcaa tcttctgatg attttgctct gattgtgaat
1381 gcacccaatc atgaagggat tcaagccgga gtcaacaggt tttatcgaac ctgtaagcta
1441 cttggaatta atatgagcaa gaaaaagtct tacataaaca gaacaggtac atttgaattc
1501 acaagttttt tctatcgtta tgggtttgtt gccaatttca gcatggagct tcccagcttt
1561 ggggtgtctg ggatcaacga gtctgcggac atgagtattg gagttactgt catcaaaaac
1621 aatatgataa acaatgatct tggtccagca accgctcaaa tggcccttca gctgttcatc
1681 aaagattaca ggtacacgta ccggtgccat agaggtgaca cacaaataca aacccgaaga
1741 tcatttgaaa taaagaaact gtgggagcaa acccattcca aagctggact gctggtctcc
1801 gacggaggcc caaatttata caacattaga aatctccaca ttcctgaagt ctgcttgaaa
1861 tgggaattaa tggatgagga ttaccagggg cgtttatgca acccactgaa cccatttgtc
1921 aaccataaag acattgaatc agtgaacaat gcagtgataa tgccagcaca tggtccagcc
1981 aaaaacatgg agtatgatgc tgttgcaaca acacactcct ggatccccaa aagaaatcga
2041 tccatcttga atacaagcca aagaggaata cttgaagatg aacaaatgta ccaaaagtgc
2101 tgcaacttat ttgaaaaatt cttccccagc agttcataca gaagaccagt cgggatatcc
2161 agtatggtgg aggctatggt ttccagagcc cgaattgatg cacgaattga tttcgaatct
2221 ggaaggataa agaaagagga gttcactgag atcatgaaga tctgttccac cattgaagag
2281 ctcagacggc aaaaatagtg aatttagctt gtccttcatg aaaaaatgcc ttgtttctac
2341 t
```

FIG. 22

```
   1 agcgaaagca ggtcaattat attcaatatg gaaagaataa aagaactaag gaatctaatg
  61 tcgcagtctc gcactcgcga gatactcaca aaaaccaccg tggaccatat ggccataatc
 121 aagaagtaca catcaggaag acaggagaag aacccagcac ttaggatgaa atggatgatg
 181 gcaatgaaat atccaattac agcagacaag aggataacgg aaatgattcc tgagagaaat
 241 gagcagggac aaactttatg gagtaaaatg aatgacgccg gatcagaccg agtgatggta
 301 tcacctctgg ctgtgacatg gtggaatagg aatggaccag tgacaagtac agttcattat
 361 ccaaaaatct acaaaacttа ttttgaaaaa gtcgaaaggt taaaacatgg aacctttggc
 421 cctgtccatt ttagaaacca agtcaaaata cgtcgaagag ttgacataaa tcctggtcat
 481 gcagatctca gtgccaaaga ggcacaggat gtaatcatgg aagttgtttt ccctaacgaa
 541 gtgggagcca ggatactaac atcggaatcg caactaacga caaccaaaga gaagaaagaa
 601 gaactccagg gttgcaaaat ttctcctctg atggtggcat acatgttgga gagagaactg
 661 gtccgcaaaa cgagattcct cccagtggct ggtggaacaa gcagtgtgta cattgaagtg
 721 ttgcatttga cccaaggaac atgctgggaa cagatgtaca ctccaggagg ggaggcgagg
 781 aatgatgatg ttgatcaaag cttaattatt gctgctagaa acatagtaag aagagccaca
 841 gtgtcagcag atccactagc atctttattg gagatgtgcc acagcacgca gattggtgga
 901 ataaggatgg taaacatcct taggcagaac ccaacagaag agcaagccgt ggatatttgc
 961 aaggctgcaa tgggactgag aattagctca tccttcagtt ttggtggatt cacatttaag
1021 agaacaagcg gatcatcagt caagagagag gaagaggtgc ttacgggcaa tcttcagaca
1081 ttgaagataa gagtgcatga gggatatgaa gagttcacaa tggttgggag aagagcaaca
1141 gctatactca gaaaagcaac caggagattg attcagctga tagtgagtgg gagggacgaa
1201 cagtcgattg ccgaagcaat aattgtggcc atggtatttt cacaagagga ttgtatgata
1261 aaagcagtta gaggtgacct gaatttcgtc aatagggcga atcagcgatt gaaccccatg
1321 caccaacttt tgagacattt tcagaaggat gcaaaggcgc tctttcaaaa ttggggaatt
1381 gaatccatcg acaatgtgat gggaatgatc gggatattgc ccgacatgac tccaagcacc
1441 gagatgtcaa tgagaggagt gagaatcagc aaaatggggg tagatgagta ttccagcgcg
1501 gagaagatag tggtgagcat tgaccgtttt ttgagagtta gggaccaacg tgggaatgta
1561 ctactgtctc ccgaggaggt cagtgaaaca cagggaacag agaaactgac aataacttac
1621 tcatcgtcaa tgatgtggga gattaatggt cctgaatcag tgttggtcaa tacctatcag
1681 tggatcatca gaaactggga aactgttaaa attcagtggt cccagaatcc tacaatgctg
1741 tacaataaaa tggaatttga gccatttcag tctttagttc caaaggccgt tagaggccaa
1801 tacagtgggt ttgtgagaac tctgttccaa caaatgaggg atgtgcttgg gacatttgat
1861 accgctcaga taataaaact tcttcccttc gcagccgctc caccaaagca aagtggaatg
1921 cagttctcct cattgactat aaatgtgagg ggatcaggaa tgagaatact tgtaaggggc
1981 aattctccaa tattcaacta caacaagacc actaaaagac tcacagttct cggaaaggat
2041 gctggccctt taactgaaga cccagatgaa ggcacagctg gagttgagtc cgcagttctg
2101 agaggattcc tcattctggg caaagaagac aggagatatg gaccagcatt aagcataaat
2161 gaactgagca accttgcgaa aggagagaag gctaatgtgc taattgggca aggagacgtg
2221 gtgttggtaa tgaaacggaa acggaactct agcatactta ctgacagcca gacagcgacc
2281 aaaagaattc ggatggccat caattagtgt cgaatagttt aaaaacgacc ttgtttctac
2341 t
```

FIG. 23

```
   1 agcaaaagca gggtagataa tcactcacag agtgacatcg aaatcatggc gaccaaaggc
  61 accaaacgat cttacgaaca gatggagact gatggagaac gccagaatgc cactgaaatc
 121 agagcatctg tcggaaaaat gattggtgga attggacgat tctacatcca aatgtgcacc
 181 gaacttaaac tcagtgatta tgagggacgg ctgattcaga acagcttaac aatagagaga
 241 atggtgctct ctgcttttga cgagaggagg aataaatatc tagaagaaca tcccagtgcg
 301 gggaaagatc ctaagaaaac tggaggacct atatacagga gagtagatgg aaagtggatg
 361 agagaactca tcctttatga caaagaagaa ataagacgaa tctggcgcca agctaataat
 421 ggtgacgatg caacggctgg tctgactcac atgatgatct ggcactccaa tttgaatgat
 481 gcaacttacc agaggacaag agctcttgtt cgcacaggaa tggatcccag gatgtgctca
 541 ctgatgcagg gttcaaccct ccctaggagg tctggggccg caggtgctgc agtcaaagga
 601 gttggaacaa tggtgatgga attgatcaga atgatcaaac gtgggatcaa tgatcggaac
 661 ttctggaggg gtgagaatgg acggagaaca aggattgctt atgaaagaat gtgcaacatt
 721 ctcaaaggga aatttcaaac agctgcacaa agagcaatgg tggatcaagt gagagagagc
 781 cggaatccag gaaatgctga gttcgaagat ctcatctttc tagcacggtc tgcactcata
 841 ttgagagggt cagttgctca caagtcctgc ctgcctgcct gtgtgtatgg acctgccgta
 901 gccagtggat acgactttga aagagaggga tactctctag tcggaataga ccctttcaga
 961 ctgcttcaaa acagccaagt atacagccta atcagaccaa atgagaatcc agcacacaag
1021 agtcaactgg tgtggatggc atgccattct gctgcatttg aagatctaag agtatcaagc
1081 ttcatcagag ggacgaaagt ggtcccaaga gggaagcttt ccactagagg agttcaaatt
1141 gcttccaatg aaaacatgga gactatggaa tcaagtaccc ttgaactgag aagcagatac
1201 tgggccataa ggaccagaag tggagggaac accaatcaac agagggcttc ctcgggccaa
1261 atcagcatac aacctacgtt ctcagtacag agaaatctcc cttttgacag accaaccatt
1321 atggcagcat tcactgggaa tacagagggg agaacatctg acatgagaac cgaaatcata
1381 aggctgatgg aaagtgcaag accagaagat gtgtctttcc aggggcgggg agtcttcgag
1441 ctctcggacg aaaaggcagc gagcccgatc gtgccctcct ttgacatgag taatgaagga
1501 tcttatttct tcggagacaa tgcagaggag tacgacaatt aaagaaaaat acccttgttt
1561 ctact
```

FIG. 24

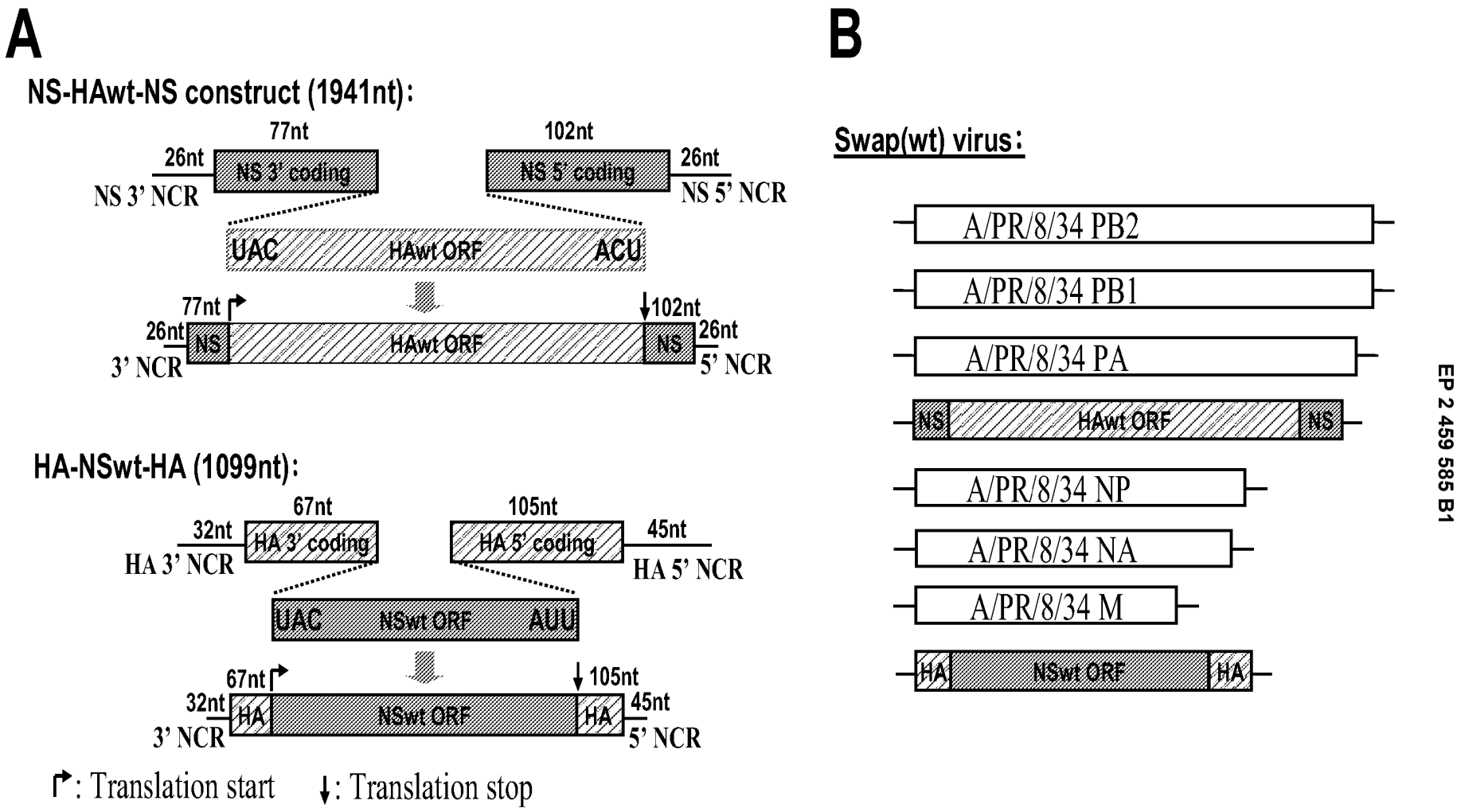

A
NS-HAwt-NS construct (1941nt):
77nt
102nt
26nt
NS 3' coding
NS 5' coding
26nt
NS 3' NCR
NS 5' NCR
UAC
HAwt ORF
ACU
77nt
102nt
26nt
NS
HAwt ORF
NS
26nt
3' NCR
5' NCR
HA-NSwt-HA (1099nt):
67nt
105nt
32nt
HA 3' coding
HA 5' coding
45nt
HA 3' NCR
HA 5' NCR
UAC
NSwt ORF
AUU
67nt
105nt
32nt
HA
NSwt ORF
HA
45nt
3' NCR
5' NCR
: Translation start
: Translation stop
B
Swap(wt) virus:
A/PR/8/34 PB2
A/PR/8/34 PB1
A/PR/8/34 PA
NS
HAwt ORF
NS
A/PR/8/34 NP
A/PR/8/34 NA
A/PR/8/34 M
HA
NSwt ORF
HA

Fig. 25A – 25B

C

Reassortant(NS) virus:

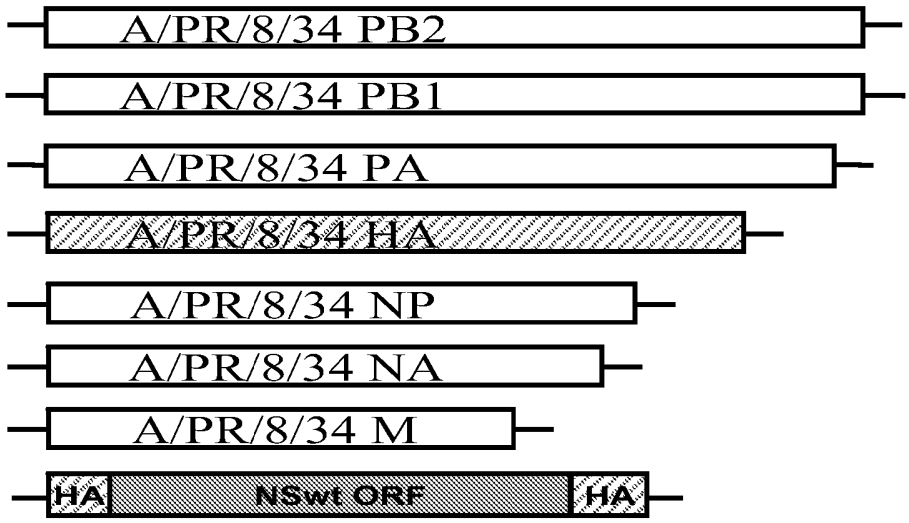


Fig. 25C

D

Reassortant(HA) virus:

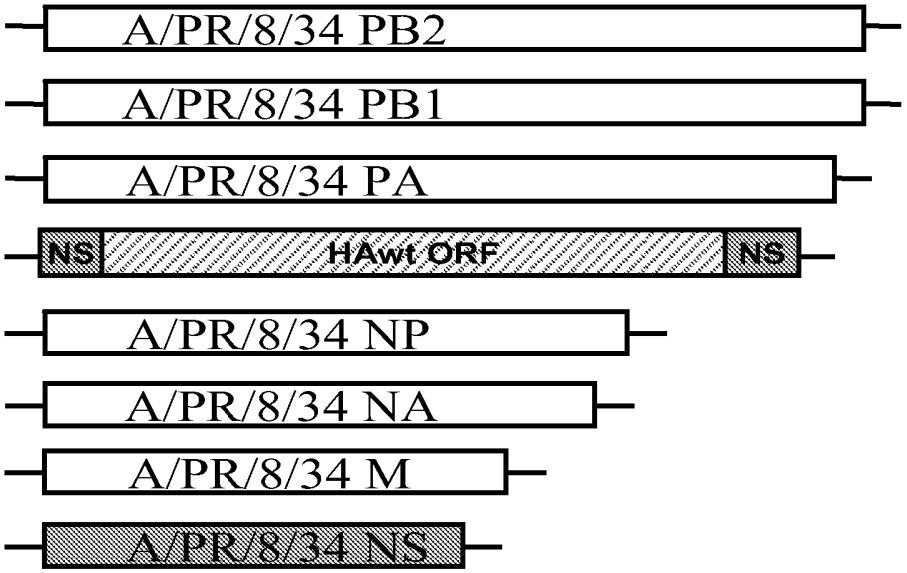


Fig. 25D

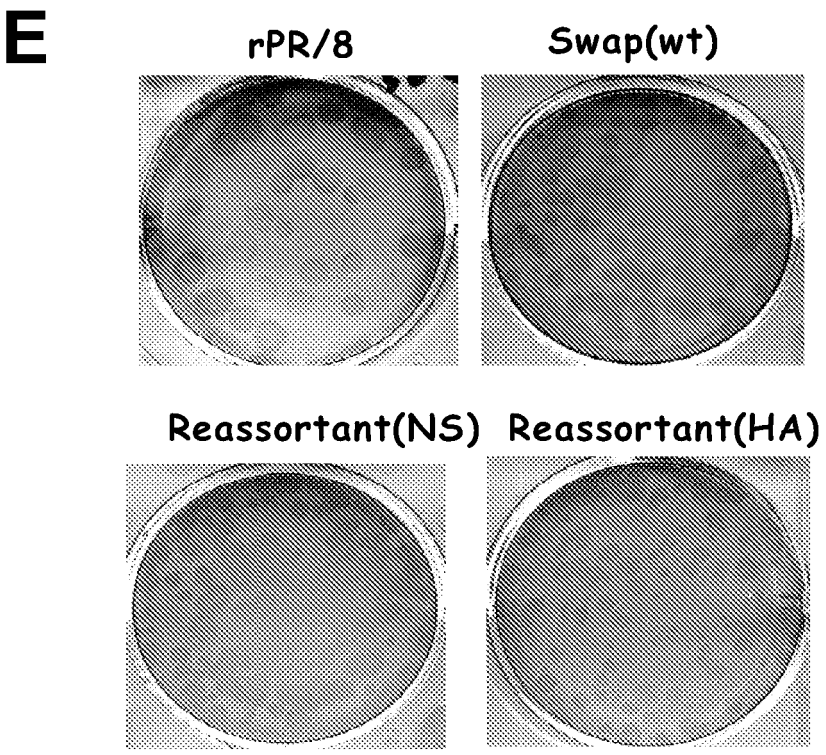


Fig. 25E

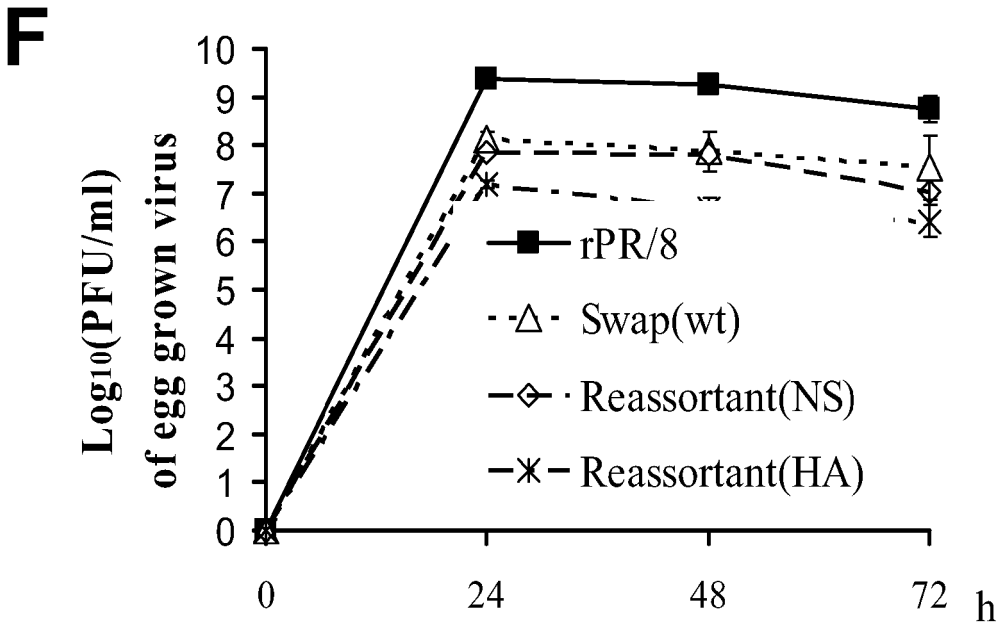
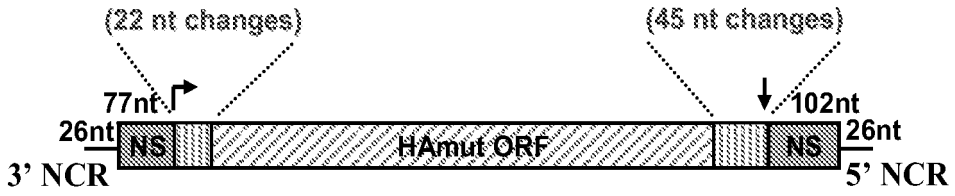
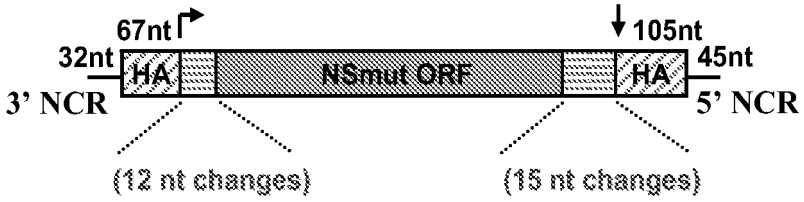


Fig. 25F

NS-HAmut-NS construct (1941nt):



HA-NSmut-HA construct (1099nt):



mut: Mutation ↱: Translation start ↓: Translation stop

Fig. 26A

Swap(mut) virus:

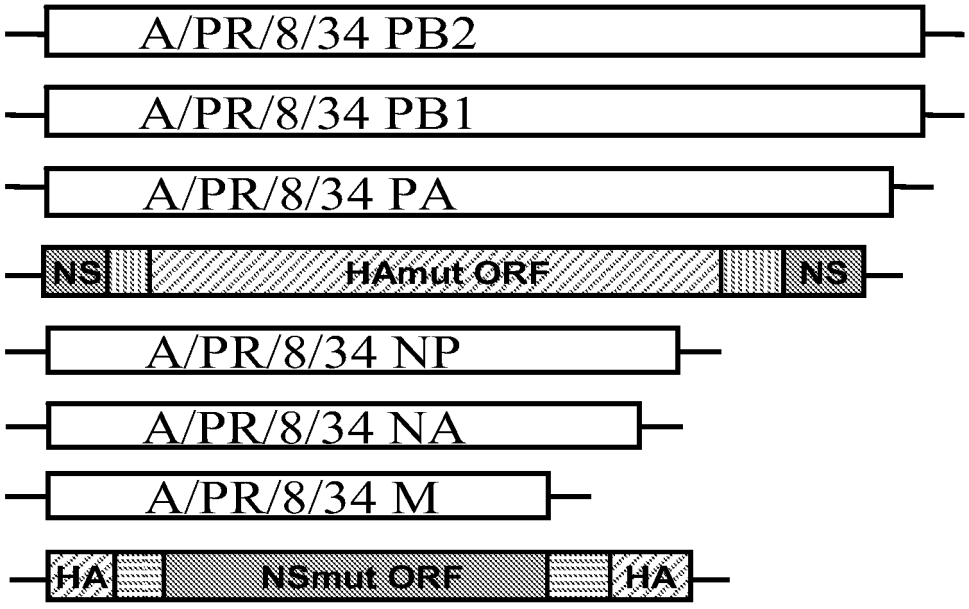


Fig. 26B

C

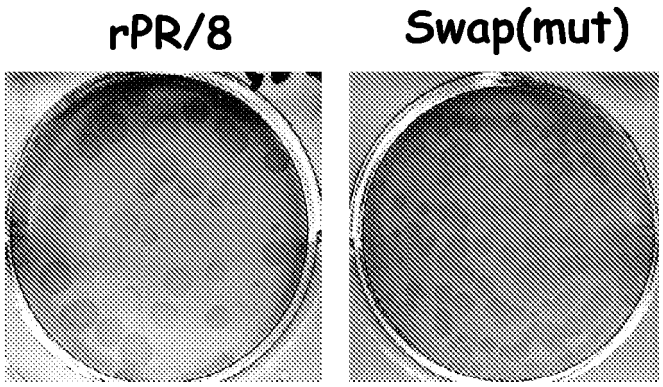


Fig. 26C

D

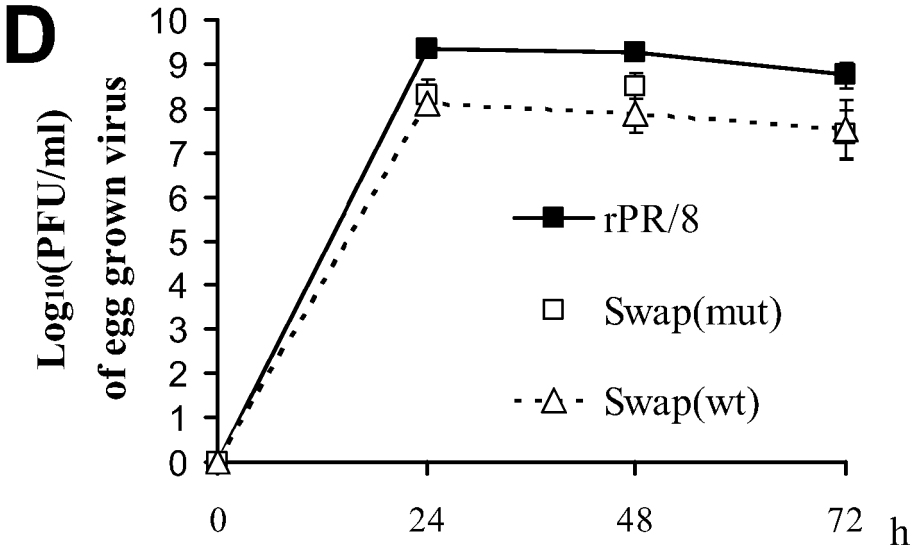


Fig. 26D

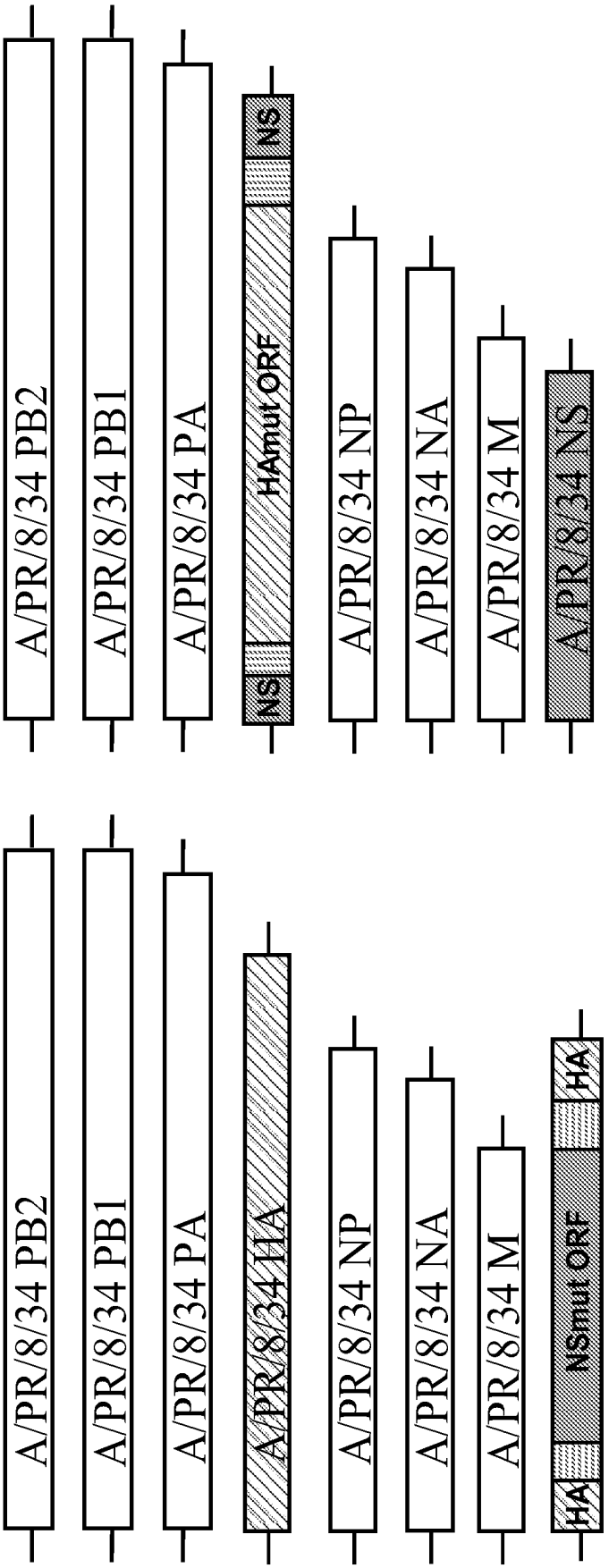

A/PR/8/34 PB2
A/PR/8/34 PB1
A/PR/8/34 PA
HAmut ORF
NS
NS
A/PR/8/34 NP
A/PR/8/34 NA
A/PR/8/34 M
A/PR/8/34 NS
No rescue
A/PR/8/34 PB2
A/PR/8/34 PB1
A/PR/8/34 PA
A/PR/8/34 HA
A/PR/8/34 NP
A/PR/8/34 NA
A/PR/8/34 M
NSmut ORF
HA
HA
No rescue

Fig. 26E

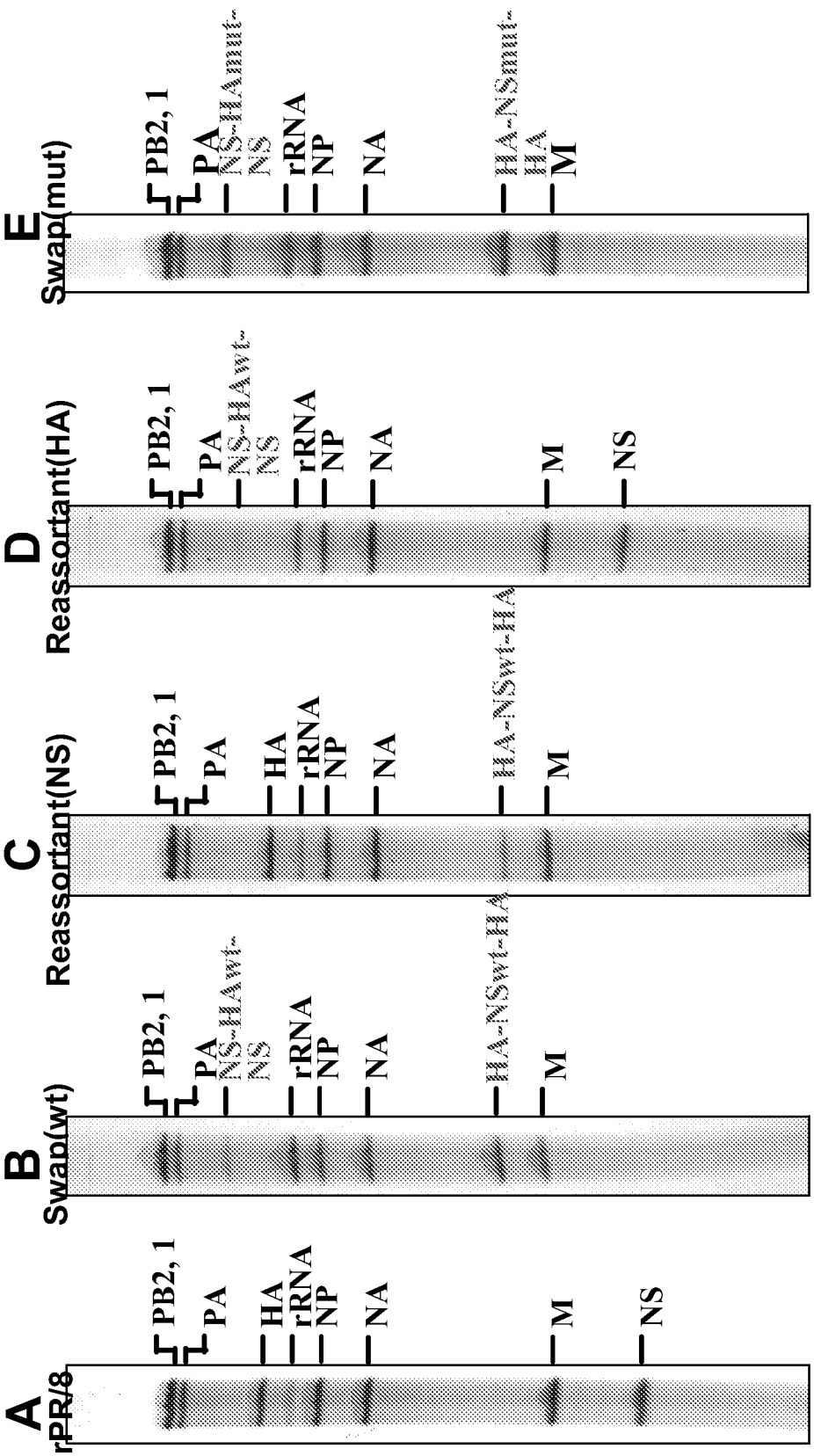

A
rPR/8
PB2, 1
PA
HA
rRNA
NP
NA
M
NS
B
Swap(wt)
PB2, 1
PA
NS-HAwt-
NS
rRNA
NP
NA
HA-NSwt-HA
M
C
Reassortant(NS)
PB2, 1
PA
HA
rRNA
NP
NA
HA-NSwt-HA
M
D
Reassortant(HA)
PB2, 1
PA
NS-HAwt-
NS
rRNA
NP
NA
M
NS
E
Swap(mut)
PB2, 1
PA
NS-HAmut-
NS
rRNA
NP
NA
HA-NSmut-
HA
M

Fig. 27

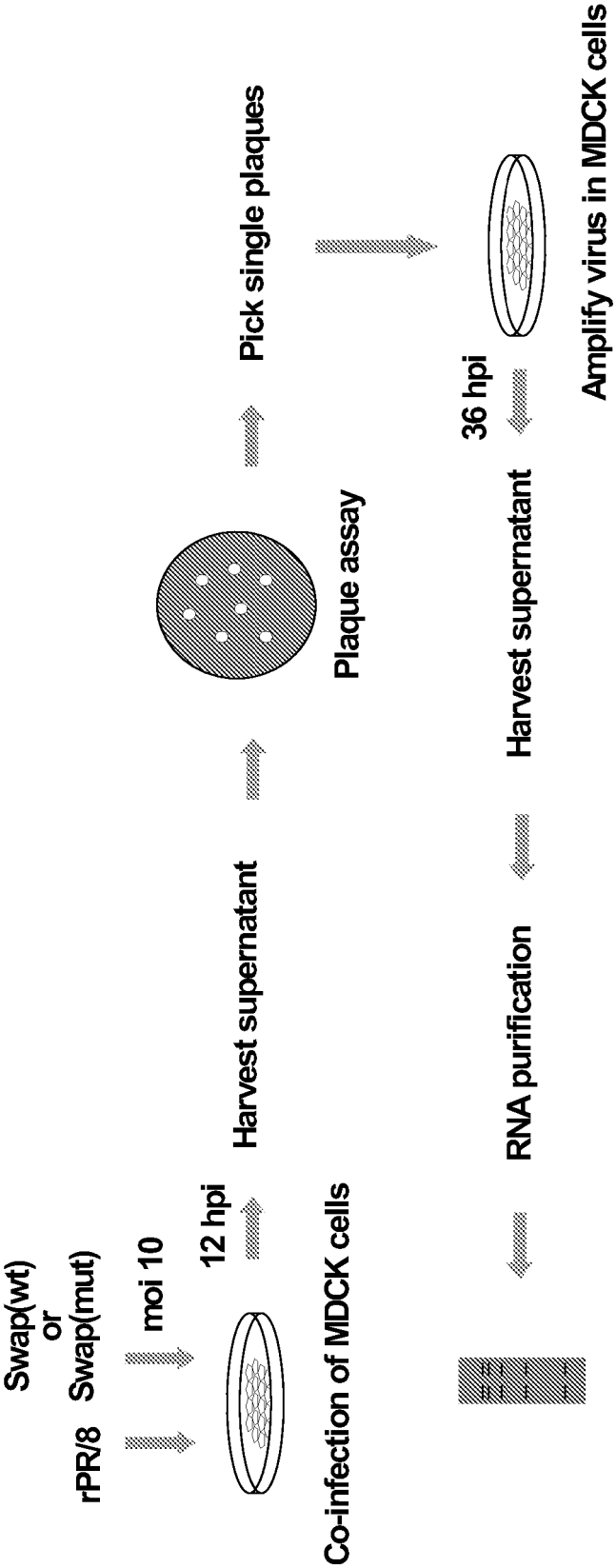

Swap(wt)
or
rPR/8 Swap(mut)
moi 10
12 hpi
Harvest supernatant
Pick single plaques
Co-infection of MDCK cells
Plaque assay
36 hpi
Harvest supernatant
Amplify virus in MDCK cells
RNA purification
RT-PCR to detect HA and NS

Fig. 28A

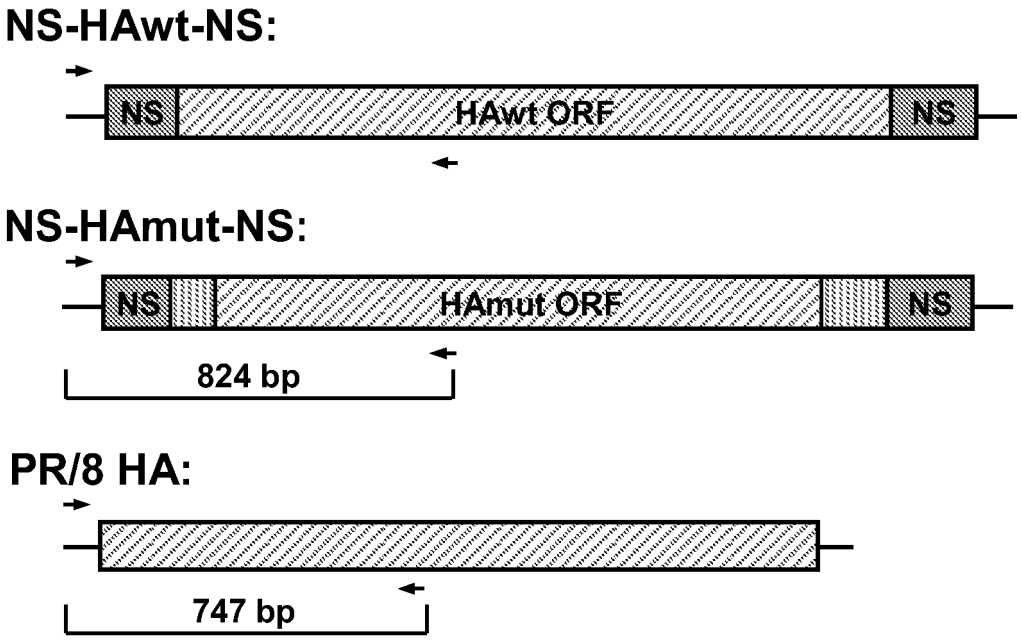


Fig. 28B

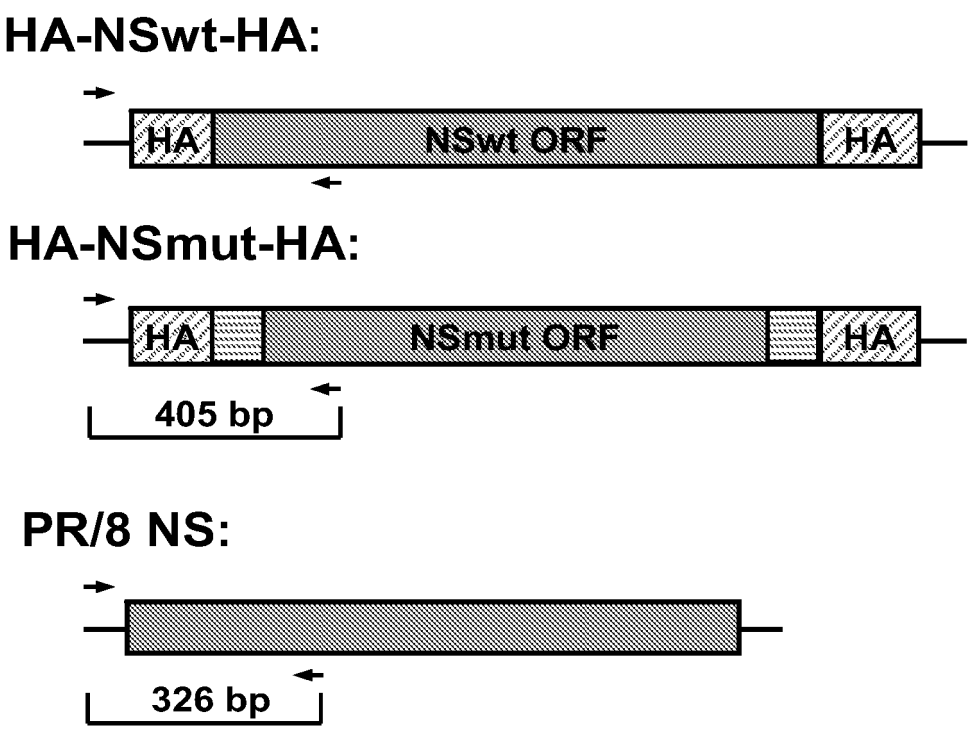


Fig. 28C

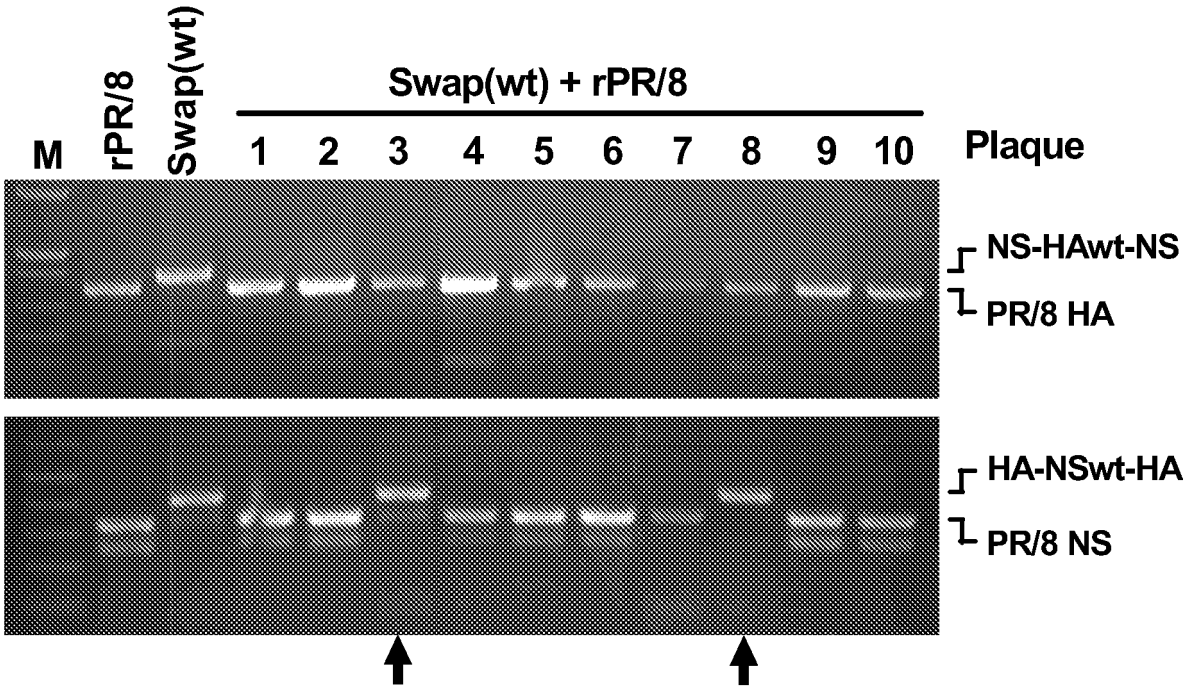


Fig. 28D

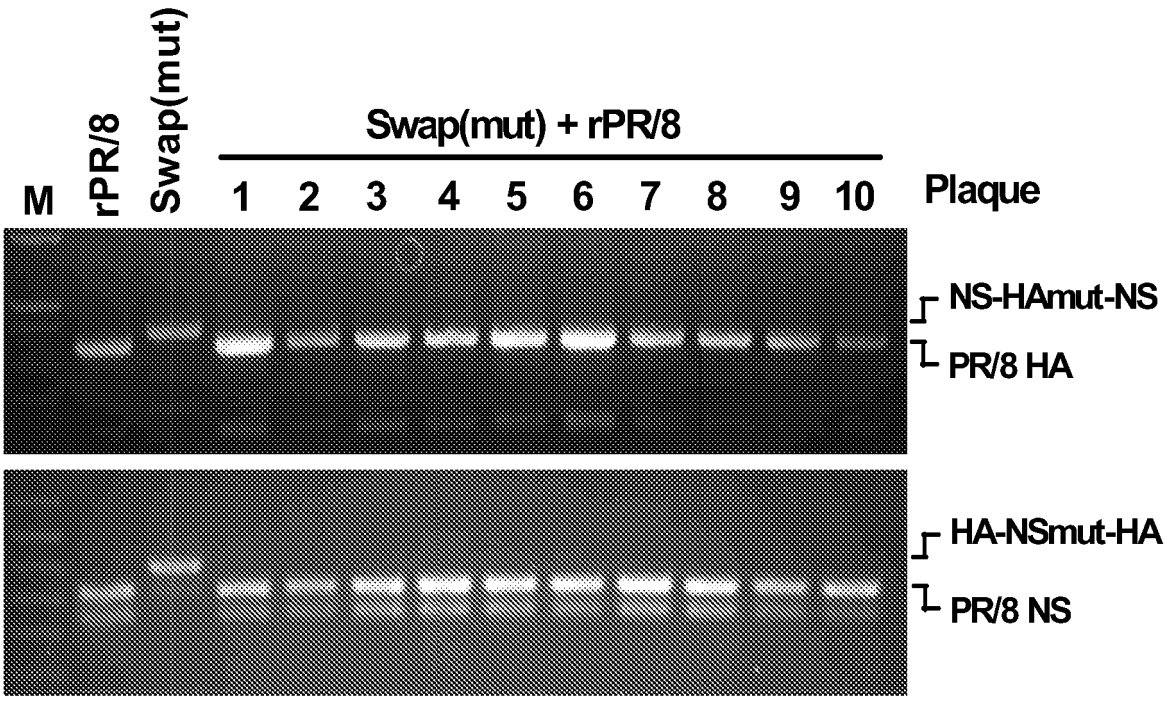


Fig. 28E

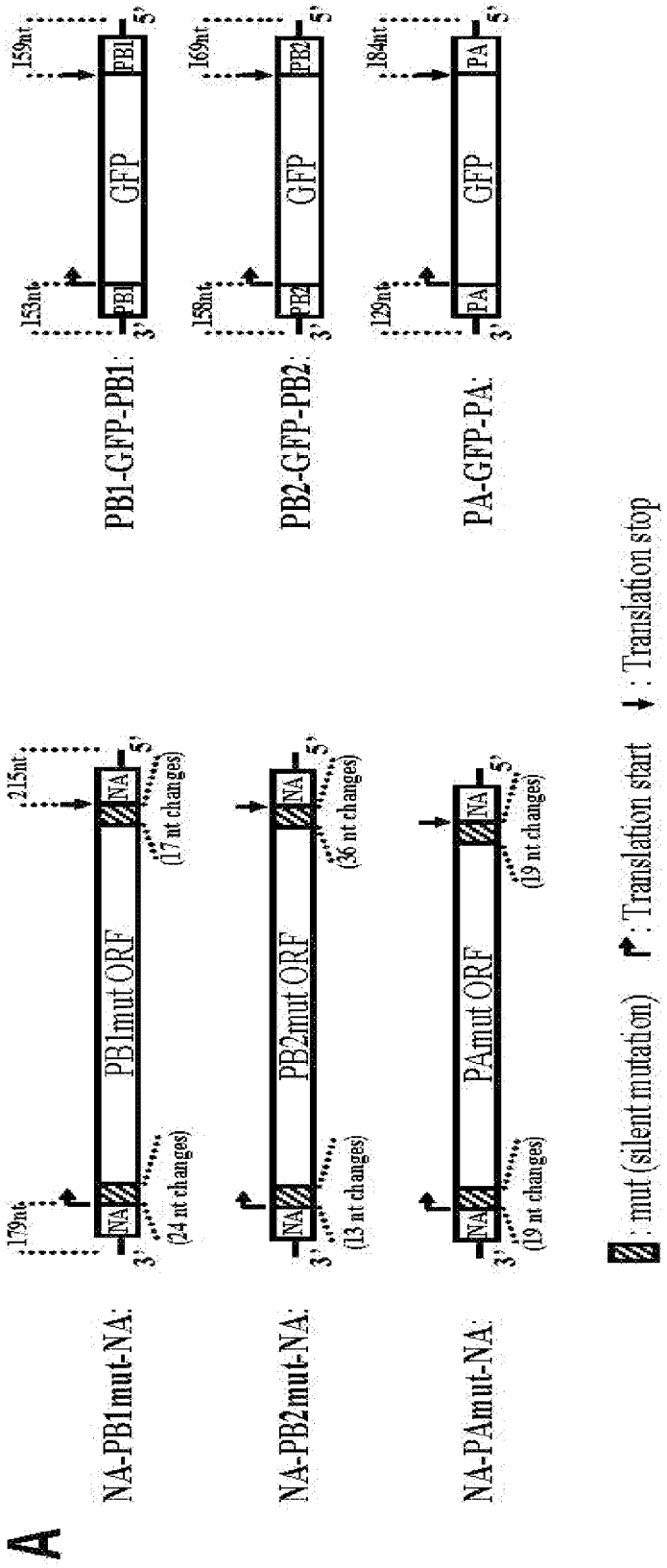

A
NA-PB1mut-NA:
179nt
215nt
NA
PB1mut ORF
NA
(24 nt changes)
(17 nt changes)
3'
5'
NA-PB2mut-NA:
NA
PB2mut ORF
NA
(13 nt changes)
(36 nt changes)
NA-PAmut-NA:
NA
PAmut ORF
NA
(19 nt changes)
(19 nt changes)
: mut (silent mutation)
: Translation start
: Translation stop
PB1-GFP-PB1:
153nt
159nt
PB1
GFP
PB1
PB2-GFP-PB2:
158nt
169nt
PB2
GFP
PB2
PA-GFP-PA:
129nt
184nt
PA
GFP
PA

Fig. 29A

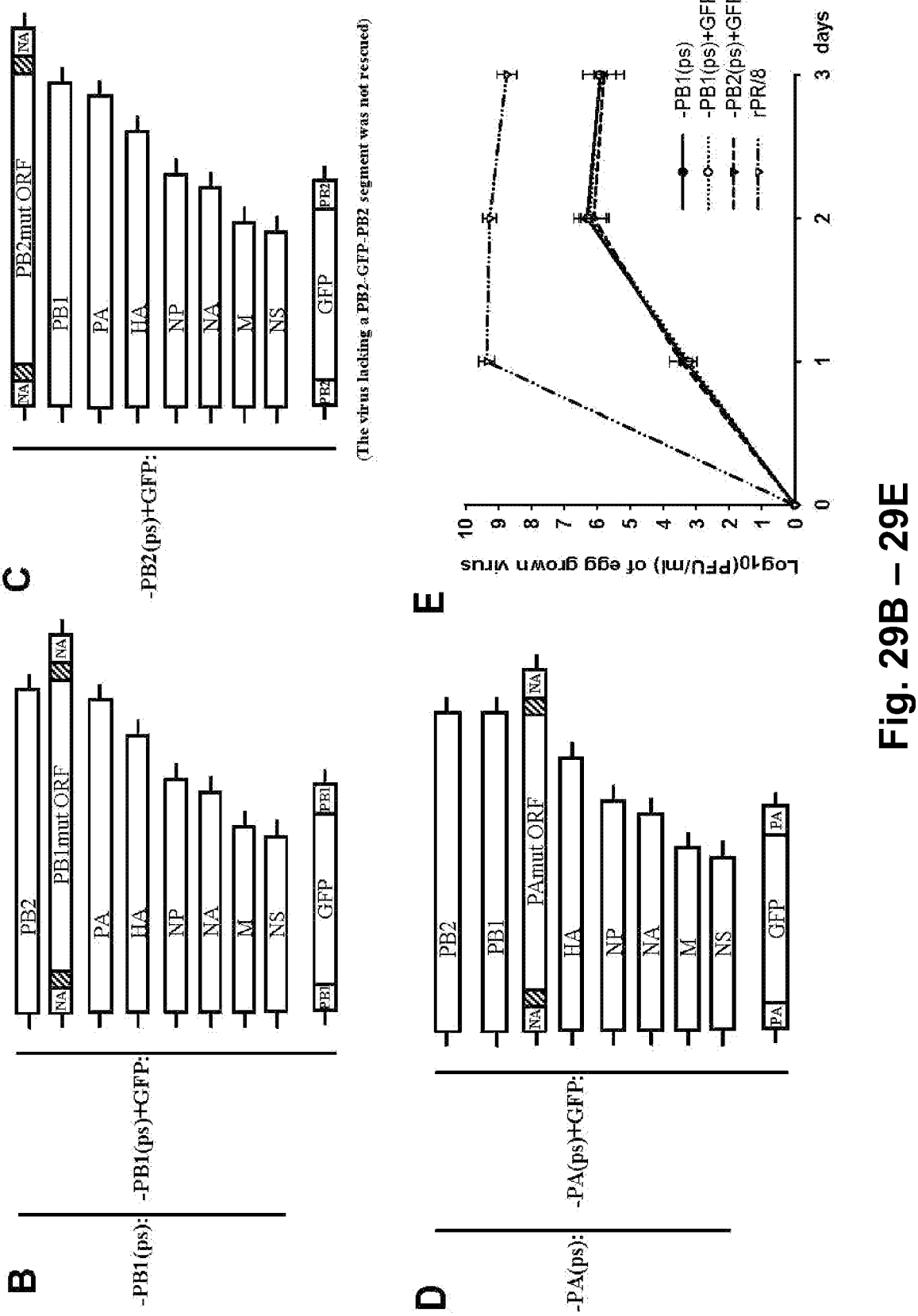

B
-PB1(ps):
-PB1(ps)+GFP:
PB2
PB1mut ORF
NA
PA
HA
NP
NA
M
NS
PB1
GFP
PB1
C
-PB2(ps)+GFP:
PB2mut ORF
NA
PB1
PA
HA
NP
NA
M
NS
PB2
GFP
PB2
(The virus lacking a PB2-GFP-PB2 segment was not rescued)
D
-PA(ps):
-PA(ps)+GFP:
PB2
PB1
PAmut ORF
NA
HA
NP
NA
M
NS
PA
GFP
PA
E
Log10(PFU/ml) of egg grown virus
10
9
8
7
6
5
4
3
2
1
0
0
1
2
3 days
-PB1(ps)
-PB1(ps)+GFP
-PB2(ps)+GFP
rPR/8

Fig. 29B – 29E

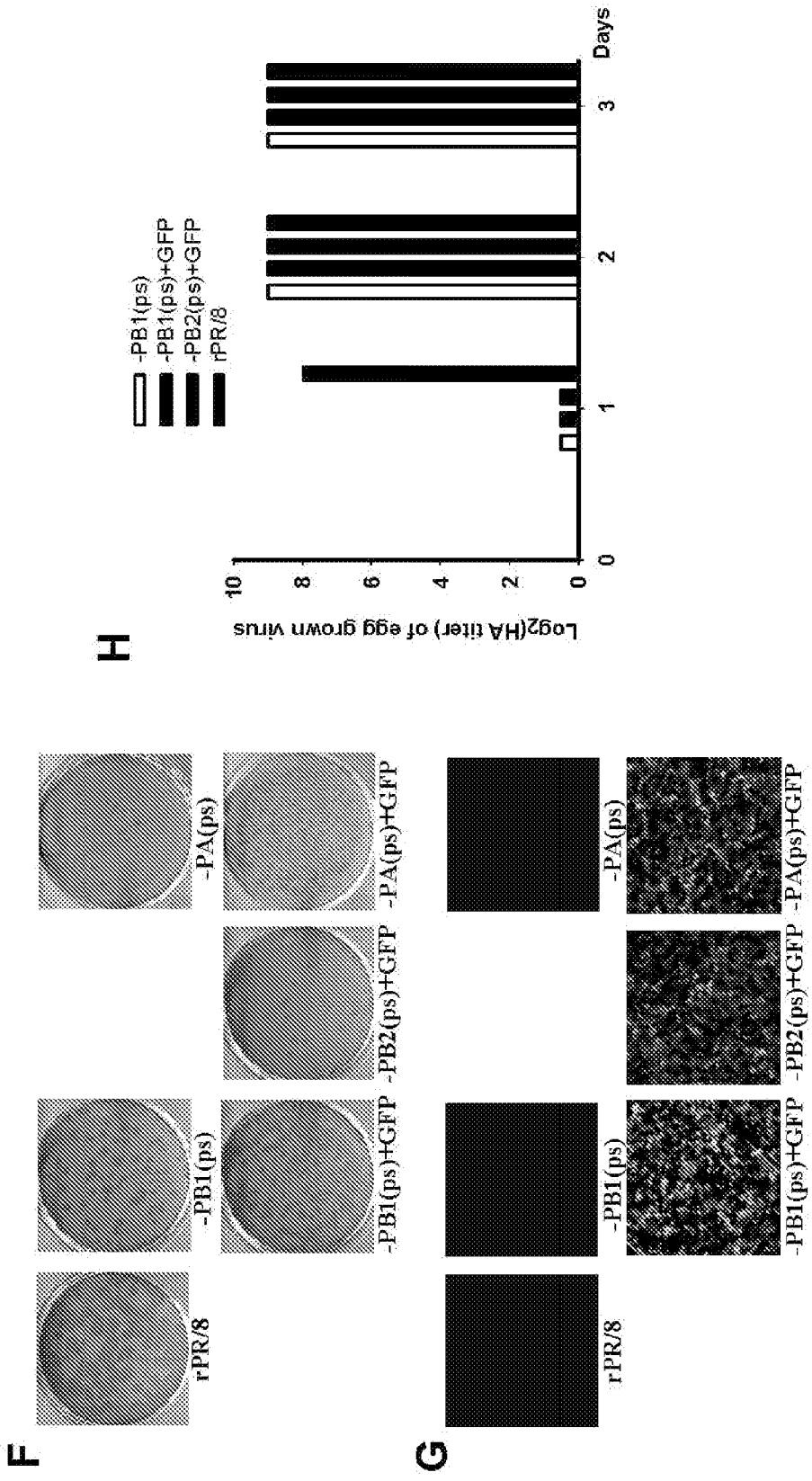

F
rPR/8
-PB1(ps)
-PB1(ps)+GFP
-PB2(ps)+GFP
-PA(ps)
-PA(ps)+GFP
G
rPR/8
-PB1(ps)
-PB1(ps)+GFP
-PB2(ps)+GFP
-PA(ps)
-PA(ps)+GFP
H
-PB1(ps)
-PB1(ps)+GFP
-PB2(ps)+GFP
rPR/8
Log2(HA titer) of egg grown virus
0
2
4
6
8
10
0
1
2
3
Days

Fig. 29F – 29H

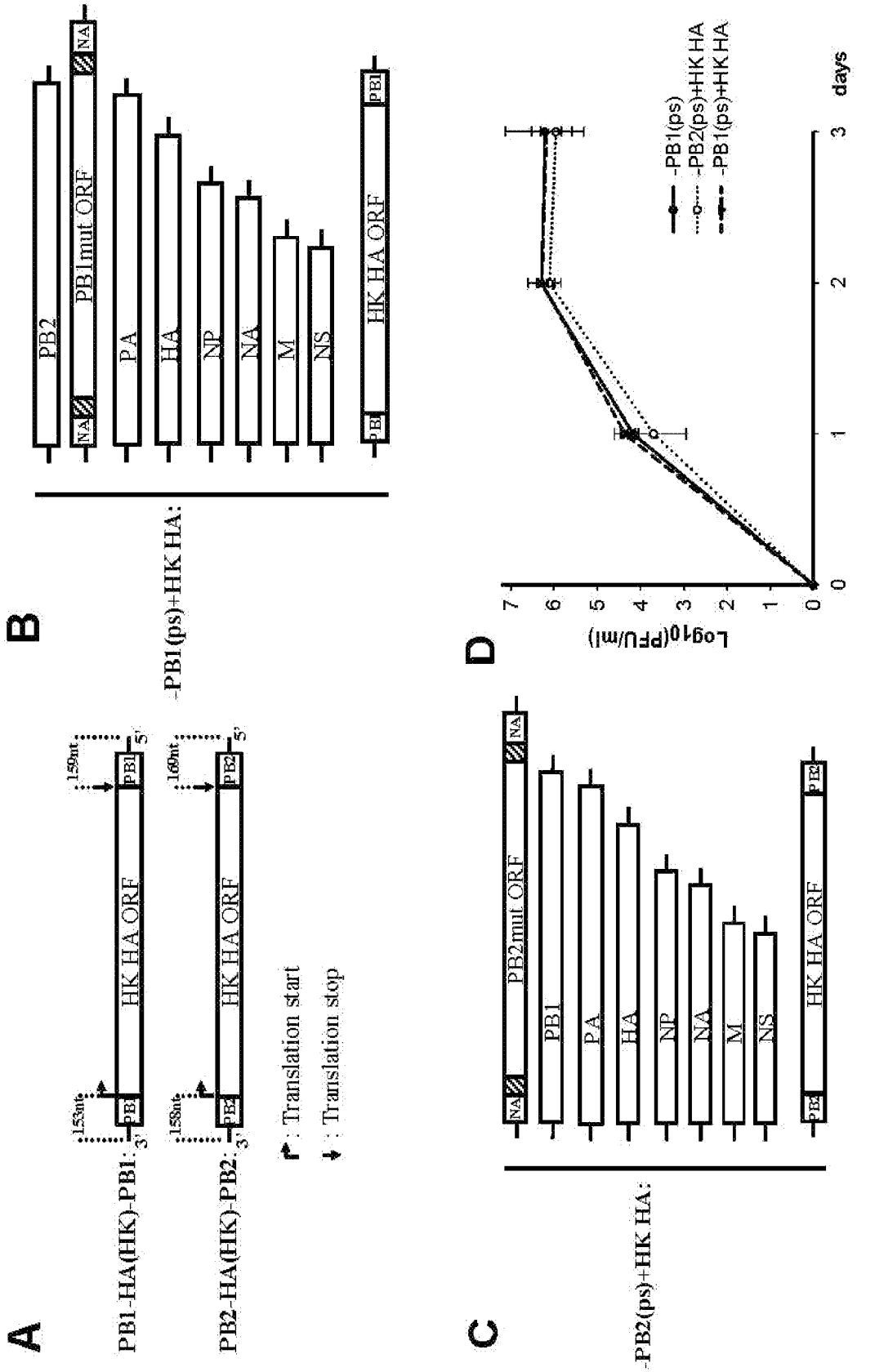

A
PB1-HA(HK)-PB1:
153nt
PB1
HK HA ORF
3'
5'
PB2-HA(HK)-PB2:
158nt
169nt
PB2
HK HA ORF
: Translation start
: Translation stop
B
-PB1(ps)+HK HA:
PB2
PB1mut ORF
NA
PA
HA
NP
NA
M
NS
HK HA ORF
PB1
C
-PB2(ps)+HK HA:
PB2mut ORF
PB1
PA
HA
NP
NA
M
NS
HK HA ORF
PB2
D
Log10(PFU/ml)
-PB1(ps)
-PB2(ps)+HK HA
-PB1(ps)+HK HA
days

Fig. 30A – 30D

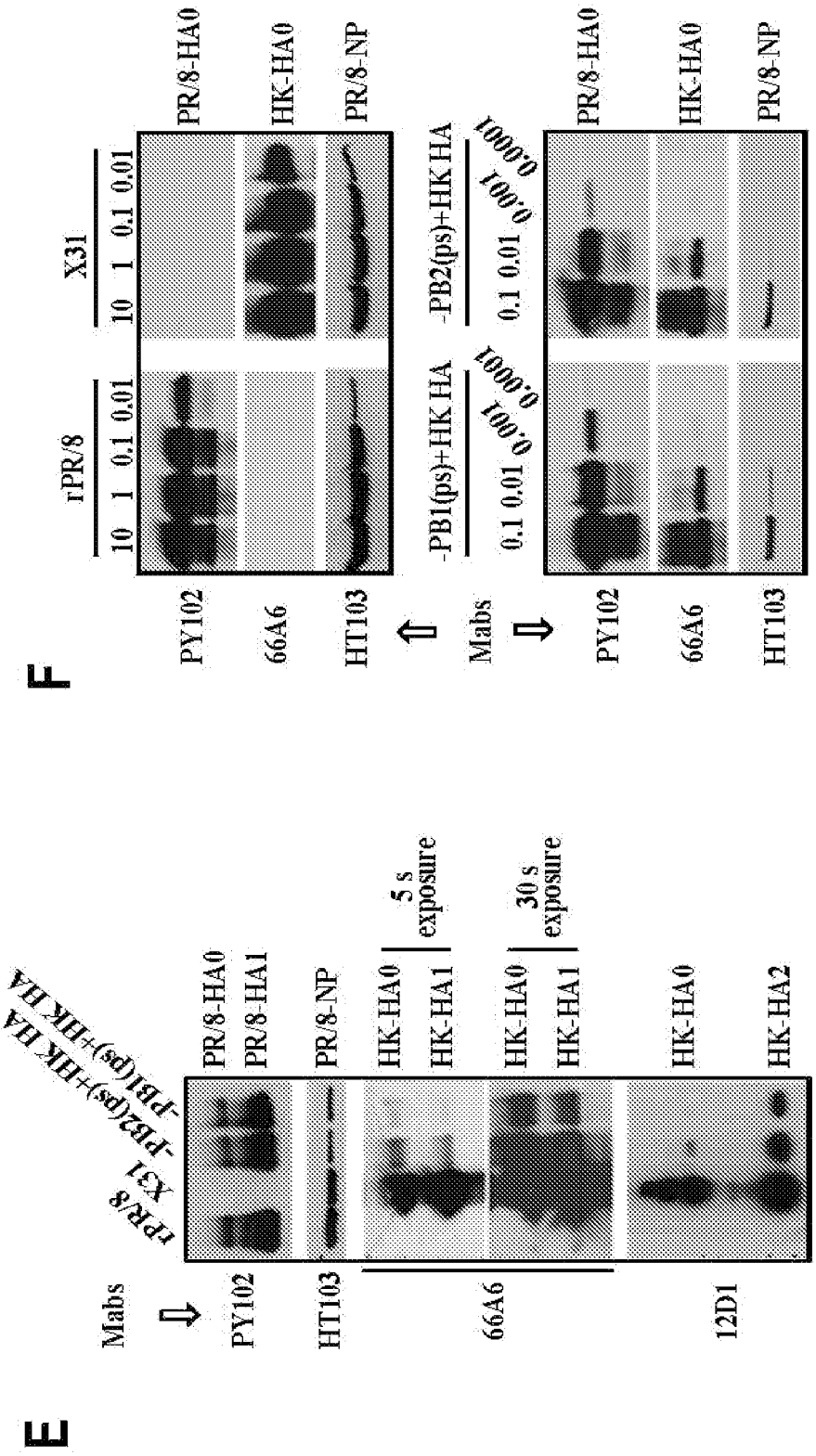

E
Mabs
PY102
HT103
66A6
12D1
rPR/8
X31
PB2(ps)+HK HA
PB1(ps)+HK HA
PR/8-HA0
PR/8-HA1
PR/8-NP
HK-HA0
HK-HA1
HK-HA2
5 s exposure
30 s exposure
F
rPR/8
X31
10 1 0,1 0,01
PY102
66A6
HT103
PR/8-HA0
HK-HA0
PR/8-NP
-PB1(ps)+HK HA
-PB2(ps)+HK HA
0,1 0,01 0,001 0,0001
Mabs

Fig. 30E – 30F

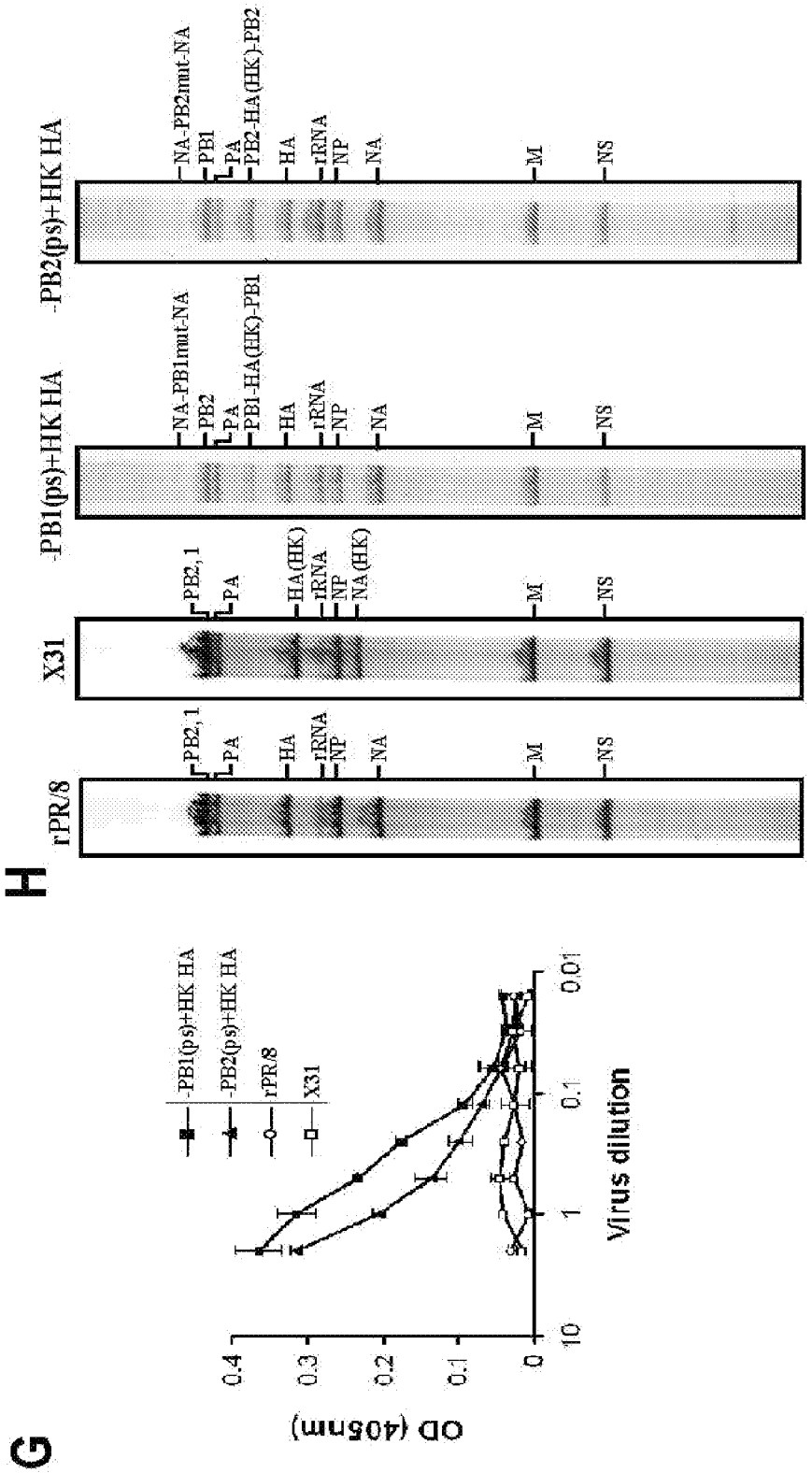
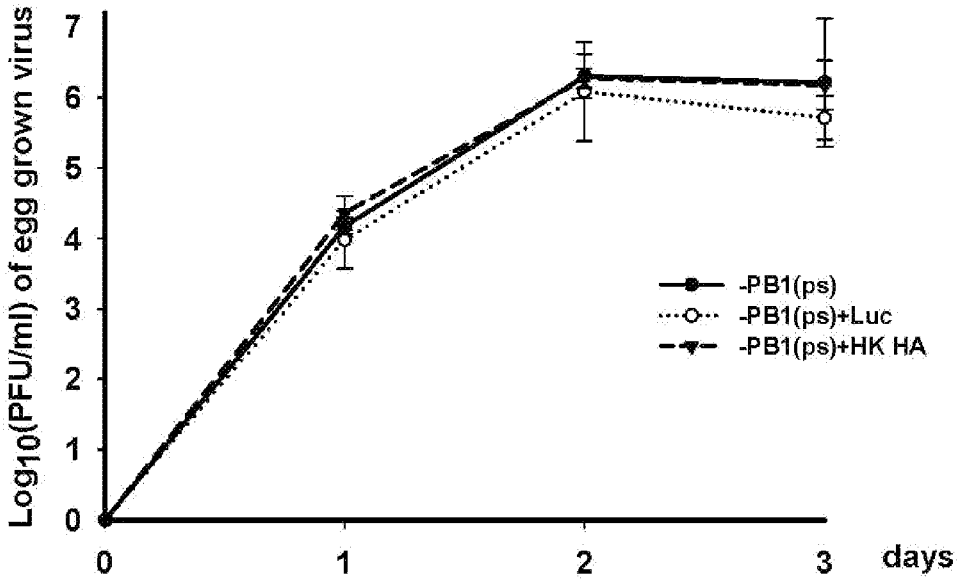

H
rPR/8
X31
-PB1(ps)+HK HA
-PB2(ps)+HK HA
PB2, 1
PA
HA
rRNA
NP
NA
M
NS
HA(HK)
NA(HK)
PB1
PB2
PB1-HA(HK)-PB1
PB2-HA(HK)-PB2
NA-PB1mut-NA
NA-PB2mut-NA
G
OD (405nm)
Virus dilution
0.4
0.3
0.2
0.1
0
10
1
0.1
0.01

Fig. 30G - 30H

A



B

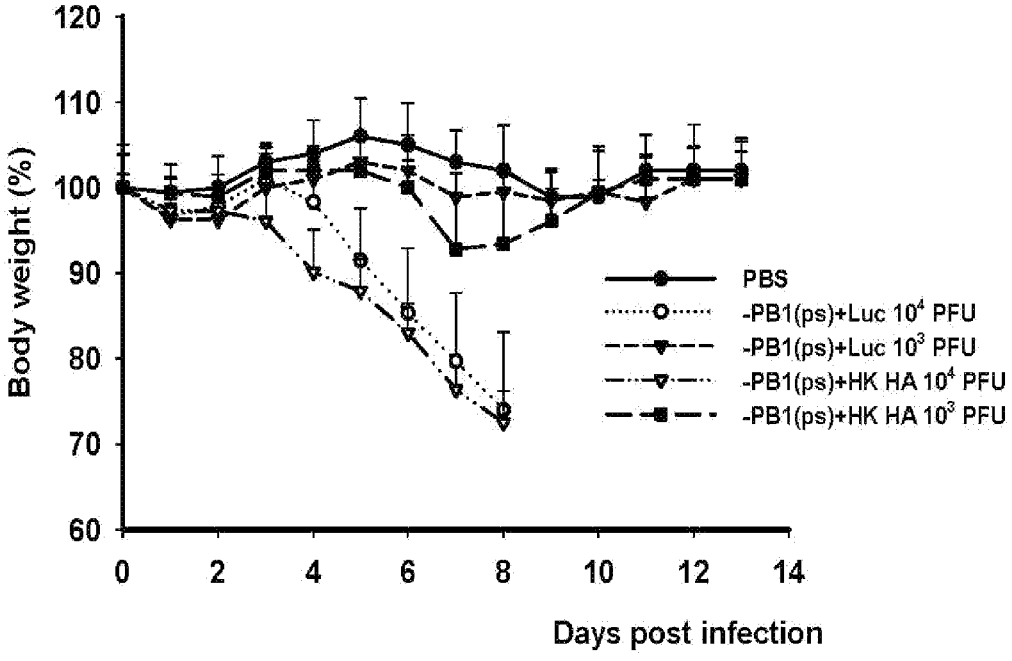
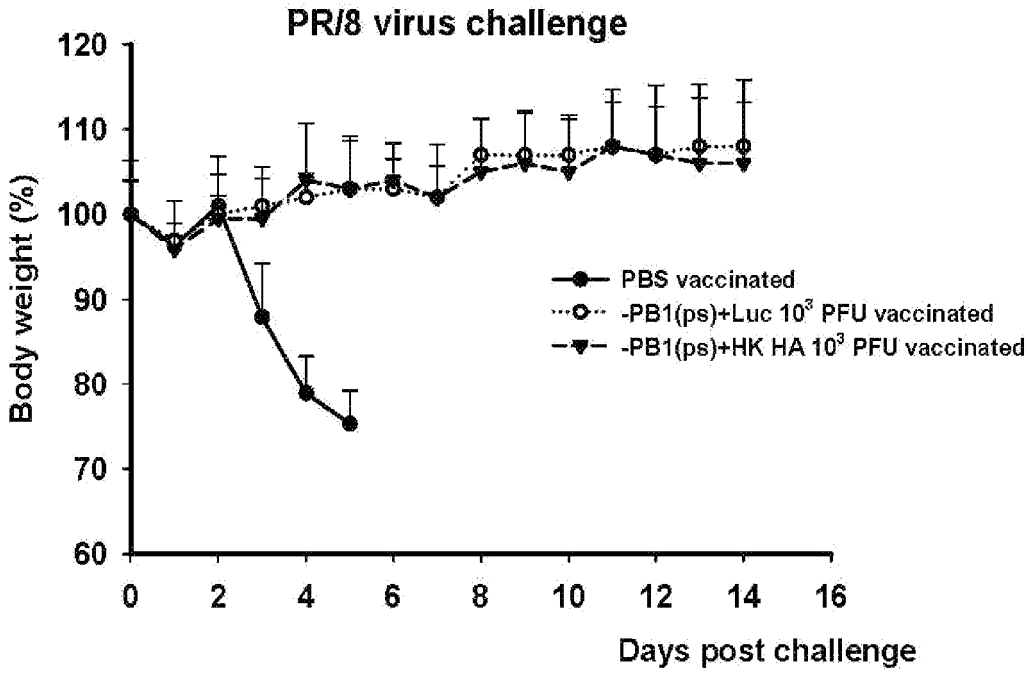


Fig. 31A – 31B

C



D

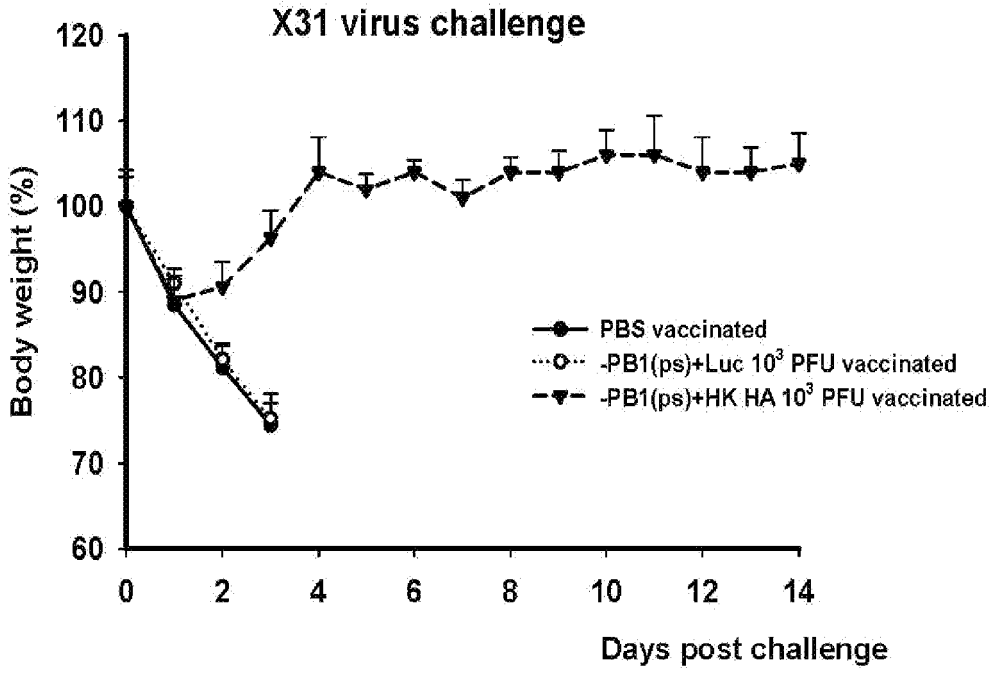


Fig. 31C – 31D

NA-PB1mut-NA:

AGCGAAAGCAGGGGTTTAAATTGAATCCAAATCAGAAAATAACAACCATTGGATCAATCTGTC
TGGTAGTCGGACTAATTAGCCTAATATTGCAAATAGGGAATATAATCTCAATTTGGATTAGCC
ATTCAATTCAAACTGGAAGTCAAAACCATACTGGAATTTGCAACCAAGCTAGCATGGACGTTA
ACCCAACTCTGTTATTTCTGAAGGTACCGGCGCAGAACGCCATCAGTACGACCTTTCCTTATA
CTGGAGACCCTCCTTACAGCCATGGGACAGGAACAGGATACACCATGGATACTGTCAACAGGA
CACATCAGTACTCAGAAAAGGGAAGATGGACAACAAACACCGAAACTGGAGCACCGCAACTCA
ACCCGATTGATGGGCCACTGCCAGAAGACAATGAACCAAGTGGTTATGCCCAAACAGATTGTG
TATTGGAAGCAATGGCTTTCCTTGAGGAATCCCATCCTGGTATTTTTGAAAACTCGTGTATTG
AAACGATGGAGGTTGTTCAGCAAACACGAGTAGACAAGCTGACACAAGGCCGACAGACCTATG
ACTGGACTCTAAATAGAAACCAACCTGCTGCAACAGCATTGGCCAACACAATAGAAGTGTTCA
GATCAAATGGCCTCACGGCCAATGAGTCTGGAAGGCTCATAGACTTCCTTAAGGATGTAATGG
AGTCAATGAAAAAAGAAGAAATGGGGATCACAACTCATTTTCAGAGAAAGAGACGGGTGAGAG
ACAATATGACTAAGAAAATGATAACACAGAGAACAATAGGTAAAAAGAAGCAGAGATTGAACA
AAAGGAGTTATCTAATTAGAGCATTGACCCTGAACACAATGACCAAAGATGCTGAGAGAGGGA
AGCTAAAACGGAGAGCAATTGCAACCCCAGGGATGCAAATAAGGGGGTTTGTATACTTTGTTG
AGACACTGGCAAGGAGTATATGTGAGAAACTTGAACAATCAGGGTTGCCAGTTGGAGGCAATG
AGAAGAAAGCAAAGTTGGCAAATGTTGTAAGGAAGATGATGACCAATTCTCAGGACACCGAAC
TTTCTTTCACCATCACTGGAGATAACACCAAATGGAACGAAAATCAGAATCCTCGGATGTTTT
TGGCCATGATCACATATATGACAAGAAATCAGCCCGAATGGTTCAGAAATGTTCTAAGTATTG
CTCCAATAATGTTCTCAAACAAAATGGCGAGACTGGGAAAAGGGTATATGTTTGAGAGCAAGA
GTATGAAACTTAGAACTCAAATACCTGCAGAAATGCTAGCAAGCATCGATTTGAAATATTTCA
ATGATTCAACAAGAAAGAAGATTGAAAAAATCCGACCGCTCTTAATAGAGGGGACTGCATCAT
TGAGCCCTGGAATGATGATGGGCATGTTCAATATGTTAAGCACTGTATTAGGCGTCTCCATCC
TGAATCTTGGACAAAAGAGATACACCAAGACTACTTACTGGTGGGATGGTCTTCAATCCTCTG
ACGATTTTGCTCTGATTGTGAATGCACCCAATCATGAAGGGATTCAAGCCGGAGTCGACAGGT
TTTATCGAACCTGTAAGCTACTTGGAATCAATATGAGCAAGAAAAAGTCTTACATAAACAGAA
CAGGTACATTTGAATTCACAAGTTTTTTCTATCGTTATGGGTTTGTTGCCAATTTCAGCATGG
AGCTCCCCAGTTTTGGGGTGTCTGGGATCAACGAGTCAGCGGACATGAGTATTGGAGTTACTG
TCATCAAAAACAATATGATAAACAATGATCTTGGTCCAGCAACAGCTCAAATGGCCCTTCAGT
TGTTCATCAAAGATTACAGGTACACGTACCGATGCCATAGAGGTGACACACAAATACAAACCC
GAAGATCATTTGAAATAAAGAAACTGTGGGAGCAAACCCGTTCCAAAGCTGGACTGCTGGTCT
CCGACGGAGGCCCAAATTTATACAACATTAGAAATCTCCACATTCCTGAAGTCTGCCTAAAAT
GGGAATTGATGGATGAGGATTACCAGGGGCGTTTATGCAACCCACTGAACCCATTTGTCAGCC
ATAAAGAAATTGAATCAATGAACAATGCAGTGATGATGCCAGCACATGGTCCAGCCAAAAACA
TGGAGTATGATGCTGTTGCAACAACACACTCCTGGATCCCCAAAAGAAATCGATCCATCTTGA
ATACAAGTCAAAGAGGAGTACTTGAAGATGAACAAATGTACCAAAGGTGCTGCAATTTATTTG
AAAAATTCTTCCCCAGCAGTTCATACAGAAGACCAGTCGGGATATCCAGTATGGTGGAGGCTA
TGGTTTCCAGAGCCCGAATTGATGCACGGATTGATTTCGAATCTGGAAGGATAAAGAAAGAAG
AGTTCACTGAGATCATGAAAATTTGCAGTACAATCGAGGAACTTCGGAGACAGAAGTAGCTCG
AGTGAGCTAACAGGGCTAGACTGTATGAGGCCGTGCTTCTGGGTTGAATTAATCAGGGGACGA
CCTAAAGAAAAAACAATCTGGACTAGTGCGAGCAGCATTTCTTTTTGTGGCGTGAATAGTGAT
ACTGTAGATTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTCAGCATTGACAAGTAGTCTGTT
CAAAAAACTCCTTGTTTCTACT

Fig. 32A

NA-PB2MUT-NA:

AGCGAAAGCAGGGGTTTAAATTGAATCCAAATCAGAAAATAACAACCATTGGATCAATCTGTC
TGGTAGTCGGACTAATTAGCCTAATATTGCAAATAGGGAATATAATCTCAATTTGGATTAGCC
ATTCAATTCAAACTGGAAGTCAAAACCATACTGGAATTTGCAACCAAGCTAGCATGGAGCGGA
TCAAGGAGTTGCGGAACTTGATGTCGCAGTCTCGCACCCGCGAGATACTCACAAAAACCACCG
TGGACCATATGGCCATAATCAAGAAGTACACATCAGGAAGACAGGAGAAGAACCCAGCACTTA
GGATGAAATGGATGATGGCAATGAAATATCCAATTACAGCAGACAAGAGGATAACGGAAATGA
TTCCTGAGAGAAATGAGCAAGGACAAACTTTATGGAGTAAAATGAATGATGCAGGATCAGACC
GAGTGATGGTATCACCTCTGGCTGTGACATGGTGGAATAGGAATGGACCAATAACAAATACAG
TTCATTATCCAAAAATCTACAAAACTTATTTTGAAAGAGTCGAAAGGCTAAAGCATGGAACCT
TTGGCCCTGTCCATTTTAGAAACCAAGTCAAAATACGTCGGAGAGTTGACATAAATCCTGGTC
ATGCAGATCTCAGTGCCAAGGAGGCACAGGATGTAATCATGGAAGTTGTTTTCCCTAACGAAG
TGGGAGCCAGGATACTAACATCGGAATCGCAACTAACGATAACCAAAGAGAAGAAAGAAGAAC
TCCAGGATTGCAAAATTTCTCCTTTGATGGTTGCATACATGTTGGAGAGAGAACTGGTCCGCA
AAACGAGATTCCTCCCAGTGGCTGGTGGAACAAGCAGTGTGTACATTGAAGTGTTGCATTTGA
CTCAAGGAACATGCTGGGAACAGATGTATACTCCAGGAGGGGAAGTGAGGAATGATGATGTTG
ATCAAAGCTTGATTATTGCTGCTAGGAACATAGTGAGAAGAGCTGCAGTATCAGCAGATCCAC
TAGCATCTTTATTGGAGATGTGCCACAGCACACAGATTGGTGGAATTAGGATGGTAGACATCC
TTAGGCAGAACCCAACAGAAGAGCAAGCCGTGGATATATGCAAGGCTGCAATGGGACTGAGAA
TTAGCTCATCCTTCAGTTTTGGTGGATTCACATTTAAGAGAACAAGCGGATCATCAGTCAAGA
GAGAGGAAGAGGTGCTTACGGGCAATCTTCAAACATTGAAGATAAGAGTGCATGAGGGATATG
AAGAGTTCACAATGGTTGGGAGAAGAGCAACAGCCATACTCAGAAAAGCAACCAGGAGATTGA
TTCAGCTGATAGTGAGTGGGAGAGACGAACAGTCGATTGCCGAAGCAATAATTGTGGCCATGG
TATTTTCACAAGAGGATTGTATGATAAAAGCAGTCAGAGGTGATCTGAATTTCGTCAATAGGG
CGAATCAGCGATTGAATCCTATGCATCAACTTTTAAGACATTTTCAGAAGGATGCGAAAGTGC
TTTTTCAAAATTGGGGAGTTGAACCTATCGACAATGTGATGGGAATGATTGGGATATTGCCAG
ACATGACTCCAAGCATCGAGATGTCAATGAGAGGAGTGAGAATCAGCAAAATGGGTGTAGATG
AGTACTCCAGCACGGAGAGGGTAGTGGTGAGCATTGACCGTTTTTTGAGAATCCGGGACCAAC
GAGGAAATGTACTACTGTCTCCCGAGGAGGTCAGTGAAACACAGGGAACAGAGAAACTGACAA
TAACTTACTCATCGTCAATGATGTGGGAGATTAATGGTCCTGAATCAGTGTTGGTCAATACCT
ATCAATGGATCATCAGAAACTGGGAAACTGTTAAAATTCAGTGGTCCCAGAACCCTACAATGC
TATACAATAAAATGGAATTTGAACCATTTCAGTCTTTAGTACCTAAGGCCATTAGAGGCCAAT
ACAGTGGGTTTGTAAGAACTCTGTTCCAACAAATGAGGGATGTGCTTGGGACATTTGATACCG
CACAGATAATAAAACTTCTTCCCTTCGCAGCCGCTCCACCAAAGCAAAGTAGAATGCAGTTCT
CCTCATTTACTGTGAATGTGAGGGGATCAGGAATGAGAATACTTGTAAGGGGCAATTCTCCTG
TATTCAACTATAACAAGGCCACGAAGAGACTCACAGTTCTCGGAAAGGATGCTGGCACTTTAA
CTGAAGACCCAGATGAAGGCACAGCTGGAGTGGAGTCCGCTGTTCTGAGGGGATTCCTCATTC
TGGGCAAAGAAGACAAGAGATATGGGCCAGCACTAAGCATCAATGAACTGAGCAACCTTGCGA
AAGGAGAGAAGGCTAATGTGCTAATTGGGCAAGGAGACGTGGTACTAGTGATGAAGAGAAAGA
GAGATAGCTCTATCTTGACGGATTCACAAACGGCAACTAAGAGGATCCGTATGGCTATTAACT
AGCTCGAGTGAGCTAACAGGGCTAGACTGTATGAGGCCGTGCTTCTGGGTTGAATTAATCAGG
GGACGACCTAAAGAAAAAACAATCTGGACTAGTGCGAGCAGCATTTCTTTTTGTGGCGTGAAT
AGTGATACTGTAGATTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTCAGCATTGACAAGTAG
TCTGTTCAAAAAACTCCTTGTTTCTACT

Fig. 32B

NA-PAmut-NA:

AGCGAAAGCAGGGGTTTAAATTGAATCCAAATCAGAAAATAACAACCATTGGATCAATCTGTC
TGGTAGTCGGACTAATTAGCCTAATATTGCAAATAGGGAATATAATCTCAATTTGGATTAGCC
ATTCAATTCAAACTGGAAGTCAAAACCATACTGGAATTTGCAACCAAGCTAGCATGGAGGACT
TCGTAAGGCAGTGTTTTAACCCAATGATCGTTGAACTCGCAGAGAAGACGATGAAGGAGTATG
GGGAGGACCTGAAAATCGAAACAAACAAATTTGCAGCAATATGCACTCACTTGGAAGTATGCT
TCATGTATTCAGATTTTCACTTCATCAATGAGCAAGGCGAGTCAATAATCGTAGAACTTGGTG
ATCCAAATGCACTTTTGAAGCACAGATTTGAAATAATCGAGGGAAGAGATCGCACAATGGCCT
GGACAGTAGTAAACAGTATTTGCAACACTACAGGGGCTGAGAAACCAAAGTTTCTACCAGATT
TGTATGATTACAAGGAGAATAGATTCATCGAAATTGGAGTAACAAGGAGAGAAGTTCACATAT
ACTATCTGGAAAAGGCCAATAAAATTAAATCTGAGAAAACACACATCCACATTTTCTCGTTCA
CTGGGGAAGAAATGGCCACAAAGGCAGACTACACTCTCGATGAAGAAAGCAGGGCTAGGATCA
AAACCAGACTATTCACCATAAGACAAGAAATGGCCAGCAGAGGCCTCTGGGATTCCTTTCGTC
AGTCCGAGAGAGGAGAAGAGACAATTGAAGAAAGGTTTGAAATCACAGGAACAATGCGCAAGC
TTGCCGACCAAAGTCTCCCGCCGAACTTCTCCAGCCTTGAAAATTTTAGAGCCTATGTGGATG
GATTCGAACCGAACGGCTACATTGAGGGCAAGCTGTCTCAAATGTCCAAAGAAGTAAATGCTA
GAATTGAACCTTTTTTGAAAACAACACCACGACCACTTAGACTTCCGAATGGGCCTCCCTGTT
CTCAGCGGTCCAAATTCCTGCTGATGGATGCCTTAAAATTAAGCATTGAGGACCCAAGTCATG
AAGGAGAGGGAATACCGCTATATGATGCAATCAAATGCATGAGAACATTCTTTGGATGGAAGG
AACCCAATGTTGTTAAACCACACGAAAAGGGAATAAATCCAAATTATCTTCTGTCATGGAAGC
AAGTACTGGCAGAACTGCAGGACATTGAGAATGAGGAGAAAATTCCAAAGACTAAAAATATGA
AGAAAACAAGTCAGCTAAAGTGGGCACTTGGTGAGAACATGGCACCAGAAAAGGTAGACTTTG
ACGACTGTAAAGATGTAGGTGATTTGAAGCAATATGATAGTGATGAACCAGAATTGAGGTCGC
TAGCAAGTTGGATTCAGAATGAGTTTAACAAGGCATGCGAACTGACAGATTCAAGCTGGATAG
AGCTCGATGAGATTGGAGAAGATGTGGCTCCAATTGAACACATTGCAAGCATGAGAAGGAATT
ATTTCACATCAGAGGTGTCTCACTGCAGAGCCACAGAATACATAATGAAGGGGGTGTACATCA
ATACTGCCTTGCTTAATGCATCTTGTGCAGCAATGGATGATTTCCAATTAATTCCAATGATAA
GCAAGTGTAGAACTAAGGAGGGAAGGCGAAAGACCAACTTGTATGGTTTCATCATAAAAGGAA
GATCCCACTTAAGGAATGACACCGACGTGGTAAACTTTGTGAGCATGGAGTTTTCTCTCACTG
ACCCAAGACTTGAACCACATAAATGGGAGAAGTACTGTGTTCTTGAGATAGGAGATATGCTTA
TAAGAAGTGCCATAGGCCAGGTTTCAAGGCCCATGTTCTTGTATGTGAGAACAAATGGAACCT
CAAAAATTAAAATGAAATGGGGAATGGAGATGAGGCGTTGCCTCCTCCAGTCACTTCAACAAA
TTGAGAGTATGATTGAAGCTGAGTCCTCTGTCAAAGAGAAAGACATGACCAAAGAGTTCTTTG
AGAACAAATCAGAAACATGGCCCATTGGAGAGTCCCCCAAAGGAGTGGAGGAAAGTTCCATTG
GGAAGGTCTGCAGGACTTTATTAGCAAAGTCGGTATTCAACAGCTTGTATGCATCTCCACAAC
TAGAAGGATTTTCAGCTGAATCAAGAAAACTGCTTCTTATCGTTCAGGCTCTTAGGGACAACC
TTGAACCTGGGACCTTTGATCTTGGGGGGCTATATGAAGCAATTGAGGAGTGCCTGATTAATG
ATCCCTGGGTTTTGCTTAACGCCAGCTGGTTTAATTCTTTTTTGACGCACGCGCTATCATAGC
TCGAGTGAGCTAACAGGGCTAGACTGTATGAGGCCGTGCTTCTGGGTTGAATTAATCAGGGGA
CGACCTAAAGAAAAAACAATCTGGACTAGTGCGAGCAGCATTTCTTTTTGTGGCGTGAATAGT
GATACTGTAGATTGGTCTTGGCCAGACGGTGCTGAGTTGCCATTCAGCATTGACAAGTAGTCT
GTTCAAAAAACTCCTTGTTTCTACT

Fig. 32C

PB1-GFP-PB1:

AGCGAAAGCAGGCAAACCATTTGATTGGTTGTCAATCCGACCTTACTTTTCTTAAAAGTGCCA
GCACAAATTGCTATAAGCACAACTTTCCCTTATACTGGAGACCCTCCTTACAGCCTTGGGACA
GGAACAGGATACACCTTGGTTGCTAGCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTG
GTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAG
GGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTG
CCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTAC
CCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAG
CGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGC
GACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTG
GGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAG
AACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCC
GACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTAC
CTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTG
GAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAACTCGAGCCC
GAATTGATGCACGGATTGATTTCGAATCTGGAAGGATAAAGAAAGAAGAGTTCACTGAGATCA
TGAAGATCTGTTCCACCATTGAAGAGCTCAGACGGCAAAAATAGTGAATTTAGCTTGTCCTTC
ATGAAAAAATGCCTTGTTTCTACT

Fig. 32D

PB2-GFP-PB2:

AGCGAAAGCAGGTCAATTATATTCAATTTGGAAAGAATAAAAGAACTAAGAAATCTATTGTCG
CAGTCTCGCACCCGCGAGATACTCACAAAAACCACCGTGGACCATTTGGCCATAATCAAGAAG
TACACATCAGGAAGACAGGAGAAGAAGCTAGCATGGTGAGCAAGGGCGAGGAGCTGTTCACCG
GGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCG
GCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCA
AGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCC
GCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCC
AGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCG
AGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACA
TCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGC
AGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGC
TCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACC
ACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCC
TGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAACTCG
AGAAAGGAGAGAAGGCTAATGTGCTAATTGGGCAAGGAGACGTGGTGTTGGTAATGAAACGGA
AACGGGACTCTAGCATACTTACTGACAGCCAGACAGCGACCAAAAGAATTCGGATGGCCATCA
ATTAGTGTCGAATAGTTTAAAAACGACCTTGTTTCTACT

Fig. 32E

PA-GFP-PA:

AGCGAAAGCAGGTACTGATCCAAATTGGAAGATTTTGTGCGACATTGCTTCAATCCGTTGATT
GTCGAGCTTGCGGAAAAAACATTGAAAGAGTTTGGGGAGGACCTGAAAATCGAAACAAACAAA
TTTGCAGCAATTTGCTAGCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCAT
CCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGG
CGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCC
CTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCA
CATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCAT
CTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCT
GGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAA
GCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCAT
CAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTA
CCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCAC
CCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGT
GACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAACTCGAGCCTGGGACCTT
TGATCTTGGGGGGCTATATGAAGCAATTGAGGAGTGCCTGATTAATGATCCCTGGGTTTTGCT
TAATGCTTCTTGGTTCAACTCCTTCCTTACACATGCATTGAGTTAGTTGTGGCAGTGCTACTA
TTTGCTATCCATACTGTCCAAAAAAGTACCTTGTTTCTACT

Fig. 32F

PB1-HA(HK)-PB1:

AGCGAAAGCAGGCAAACCATTTGATTGGTTGTCAATCCGACCTTACTTTTCTTAAAAGTGCCA
GCACAAATTGCTATAAGCACAACTTTCCCTTATACTGGAGACCCTCCTTACAGCCTTGGGACA
GGAACAGGATACACCTTGGTTGCTAGCATGAAGACCATCATTGCTTTGAGCTACATTTTCTGT
CTGGCTCTCGGCCAAGACCTTCCAGGAAATGACAACAGCACAGCAACGCTGTGCCTGGGACAT
CATGCGGTGCCAAACGGAACACTAGTGAAAACAATCACAGATGATCAGATTGAAGTGACTAAT
GCTACTGAGCTAGTTCAGAGCTCCTCAACGGGGAAAATATGCAACAATCCTCATCGAATCCTT
GATGGAATAGACTGCACACTGATAGATGCTCTATTGGGGGACCCTCATTGTGATGTTTTTCAA
AATGAGACATGGGACCTTTTCGTTGAACGCAGCAAAGCTTTCAGCAACTGTTACCCTTATGAT
GTGCCAGATTATGCCTCCCTTAGGTCACTAGTTGCCTCGTCAGGCACTCTGGAGTTTATCACT
GAGGGTTTCACTTGGACTGGGGTCACTCAGAATGGGGGAAGCAATGCTTGCAAAAGGGGACCT
GGTAGCGGTTTTTTCAGTAGACTGAACTGGTTGACCAAATCAGGAAGCACATATCCAGTGCTG
AACGTGACTATGCCAAACAATGACAATTTTGACAAACTATACATTTGGGGGGTTCACCACCCG
AGCACGAACCAAGAACAAACCAGCCTGTATGTTCAAGCATCAGGGAGAGTCACAGTCTCTACC
AGGAGAAGCCAGCAAACTATAATCCCGAATATCGGGTCCAGACCCTGGGTAAGGGGTCTGTCT
AGTAGAATAAGCATCTATTGGACAATAGTTAAGCCGGGAGACGTACTGGTAATTAATAGTAAT
GGGAACCTAATCGCTCCTCGGGGTTATTTCAAAATGCGCACTGGGAAAAGCTCAATAATGAGG
TCAGATGCACCTATTGATACCTGTATTTCTGAATGCATCACTCCAAATGGAAGCATTCCCAAT
GACAAGCCCTTTCAAAACGTAAACAAGATCACATATGGAGCATGCCCCAAGTATGTTAAGCAA
AACACCCTGAAGTTGGCAACAGGGATGCGGAATGTACCAGAGAAACAAACTAGAGGCCTATTC
GGCGCAATAGCAGGTTTCATAGAAAATGGTTGGGAGGGAATGATAGACGGTTGGTACGGTTTC
AGGCATCAAAATTCTGAGGGCACAGGACAAGCAGCAGATCTTAAAAGCACTCAAGCAGCCATC
GACCAAATCAATGGGAAATTGAACAGGGTAATCGAGAAGACGAACGAGAAATTCCATCAAATC
GAAAAGGAATTCTCAGAAGTAGAAGGGAGAATTCAGGACCTCGAGAAATACGTTGAAGACACT
AAAATAGATCTCTGGTCTTACAATGCGGAGCTTCTTGTCGCTCTGGAGAATCAACATACAATT
GACCTGACTGACTCGGAAATGAACAAGCTGTTTGAAAAAACAGGGAGGCAACTGAGGGAAAAT
GCTGAAGACATGGGCAATGGTTGCTTCAAAATATACCACAAATGTGACAACGCTTGCATAGAG
TCAATCAGAAATGGGACTTATGACCATGATGTATACAGAGACGAAGCATTAAACAACCGGTTT
CAGATCAAAGGTGTTGAACTGAAGTCTGGATACAAAGACTGGATCCTGTGGATTTCCTTTGCC
ATATCATGCTTTTTGCTTTGTGTTGTTTTGCTGGGGTTCATCATGTGGGCCTGCCAGAGAGGC
AACATTAGGTGCAACATTTGCATTTGACTCGAGCCCGAATTGATGCACGGATTGATTTCGAAT
CTGGAAGGATAAAGAAAGAAGAGTTCACTGAGATCATGAAGATCTGTTCCACCATTGAAGAGT
CAGACGGCAAAAATAGTGAATTTAGCTTGTCCTTCATGAAAAAATGCCTTGTTTCTACT

Fig. 32G

PB2-HA(HK)-PB2:

AGCGAAAGCAGGTCAATTATATTCAATTTGGAAAGAATAAAAGAACTAAGAAATCTATTGTCG
CAGTCTCGCACCCGCGAGATACTCACAAAAACCACCGTGGACCATTTGGCCATAATCAAGAAG
TACACATCAGGAAGACAGGAGAAGAAGCTAGCATGAAGACCATCATTGCTTTGAGCTACATTT
TCTGTCTGGCTCTCGGCCAAGACCTTCCAGGAAATGACAACAGCACAGCAACGCTGTGCCTGG
GACATCATGCGGTGCCAAACGGAACACTAGTGAAAACAATCACAGATGATCAGATTGAAGTGA
CTAATGCTACTGAGCTAGTTCAGAGCTCCTCAACGGGGAAAATATGCAACAATCCTCATCGAA
TCCTTGATGGAATAGACTGCACACTGATAGATGCTCTATTGGGGGACCCTCATTGTGATGTTT
TTCAAAATGAGACATGGGACCTTTTCGTTGAACGCAGCAAAGCTTTCAGCAACTGTTACCCTT
ATGATGTGCCAGATTATGCCTCCCTTAGGTCACTAGTTGCCTCGTCAGGCACTCTGGAGTTTA
TCACTGAGGGTTTCACTTGGACTGGGGTCACTCAGAATGGGGGAAGCAATGCTTGCAAAAGGG
GACCTGGTAGCGGTTTTTTCAGTAGACTGAACTGGTTGACCAAATCAGGAAGCACATATCCAG
TGCTGAACGTGACTATGCCAAACAATGACAATTTTGACAAACTATACATTTGGGGGGTTCACC
ACCCGAGCACGAACCAAGAACAAACCAGCCTGTATGTTCAAGCATCAGGGAGAGTCACAGTCT
CTACCAGGAGAAGCCAGCAAACTATAATCCCGAATATCGGGTCCAGACCCTGGGTAAGGGGTC
TGTCTAGTAGAATAAGCATCTATTGGACAATAGTTAAGCCGGGAGACGTACTGGTAATTAATA
GTAATGGGAACCTAATCGCTCCTCGGGGTTATTTCAAAATGCGCACTGGGAAAAGCTCAATAA
TGAGGTCAGATGCACCTATTGATACCTGTATTTCTGAATGCATCACTCCAAATGGAAGCATTC
CCAATGACAAGCCCTTTCAAAACGTAAACAAGATCACATATGGAGCATGCCCCAAGTATGTTA
AGCAAAACACCCTGAAGTTGGCAACAGGGATGCGGAATGTACCAGAGAAACAAACTAGAGGCC
TATTCGGCGCAATAGCAGGTTTCATAGAAAATGGTTGGGAGGGAATGATAGACGGTTGGTACG
GTTTCAGGCATCAAAATTCTGAGGGCACAGGACAAGCAGCAGATCTTAAAAGCACTCAAGCAG
CCATCGACCAAATCAATGGGAAATTGAACAGGGTAATCGAGAAGACGAACGAGAAATTCCATC
AAATCGAAAAGGAATTCTCAGAAGTAGAAGGGAGAATTCAGGACCTCGAGAAATACGTTGAAG
ACACTAAAATAGATCTCTGGTCTTACAATGCGGAGCTTCTTGTCGCTCTGGAGAATCAACATA
CAATTGACCTGACTGACTCGGAAATGAACAAGCTGTTTGAAAAAACAGGGAGGCAACTGAGGG
AAAATGCTGAAGACATGGGCAATGGTTGCTTCAAAATATACCACAAATGTGACAACGCTTGCA
TAGAGTCAATCAGAAATGGGACTTATGACCATGATGTATACAGAGACGAAGCATTAAACAACC
GGTTTCAGATCAAAGGTGTTGAACTGAAGTCTGGATACAAAGACTGGATCCTGTGGATTTCCT
TTGCCATATCATGCTTTTTGCTTTGTGTTGTTTTGCTGGGGTTCATCATGTGGGCCTGCCAGA
GAGGCAACATTAGGTGCAACATTTGCATTTGACTCGAGAAAGGAGAGAAGGCTAATGTGCTAA
TTGGGCAAGGAGACGTGGTGTTGGTAATGAAACGGAAACGGGACTCTAGCATACTTACTGACA
GCCAGACAGCGACCAAAAGAATTCGGATGGCCATCAATTAGTGTCGAATAGTTTAAAAACGAC
CTTGTTTCTACT

Fig. 32H

PB1-Luc-PB1:

AGCGAAAGCAGGCAAACCATTTGATTGGTTGTCAATCCGACCTTACTTTTCTTAAAAGTGCCA
GCACAAATTGCTATAAGCACAACTTTCCCTTATACTGGAGACCCTCCTTACAGCCTTGGGACA
GGAACAGGATACACCTTGGTTGCTAGCATGACTTCGAAAGTTTATGATCCAGAACAAAGGAAA
CGGATGATAACTGGTCCGCAGTGGTGGGCCAGATGTAAACAAATGAATGTTCTTGATTCATTT
ATTAATTATTATGATTCAGAAAAACATGCAGAAAATGCTGTTATTTTTTTACATGGTAACGCG
GCCTCTTCTTATTTATGGCGACATGTTGTGCCACATATTGAGCCAGTAGCGCGGTGTATTATA
CCAGACCTTATTGGTATGGGCAAATCAGGCAAATCTGGTAATGGTTCTTATAGGTTACTTGAT
CATTACAAATATCTTACTGCATGGTTTGAACTTCTTAATTTACCAAAGAAGATCATTTTTGTC
GGCCATGATTGGGGTGCTTGTTTGGCATTTCATTATAGCTATGAGCATCAAGATAAGATCAAA
GCAATAGTTCACGCTGAAAGTGTAGTAGATGTGATTGAATCATGGGATGAATGGCCTGATATT
GAAGAAGATATTGCGTTGATCAAATCTGAAGAAGGAGAAAAAATGGTTTTGGAGAATAACTTC
TTCGTGGAAACCATGTTGCCATCAAAAATCATGAGAAAGTTAGAACCAGAAGAATTTGCAGCA
TATCTTGAACCATTCAAAGAGAAAGGTGAAGTTCGTCGTCCAACATTATCATGGCCTCGTGAA
ATCCCGTTAGTAAAAGGTGGTAAACCTGACGTTGTACAAATTGTTAGGAATTATAATGCTTAT
CTACGTGCAAGTGATGATTTACCAAAAATGTTTATTGAATCGGACCCAGGATTCTTTTCCAAT
GCTATTGTTGAAGGTGCCAAGAAGTTTCCTAATACTGAATTTGTCAAAGTAAAAGGTCTTCAT
TTTTCGCAAGAAGATGCACCTGATGAAATGGGAAAATATATCAAATCGTTCGTTGAGCGAGTT
CTCAAAAATGAACAATAACTCGAGCCCGAATTGATGCACGGATTGATTTCGAATCTGGAAGGA
TAAAGAAAGAAGAGTTCACTGAGATCATGAAGATCTGTTCCACCATTGAAGAGCTCAGACGGC
AAAAATAGTGAATTTAGCTTGTCCTTCATGAAAAAATGCCTTGTTTCTACT

Fig. 32 I

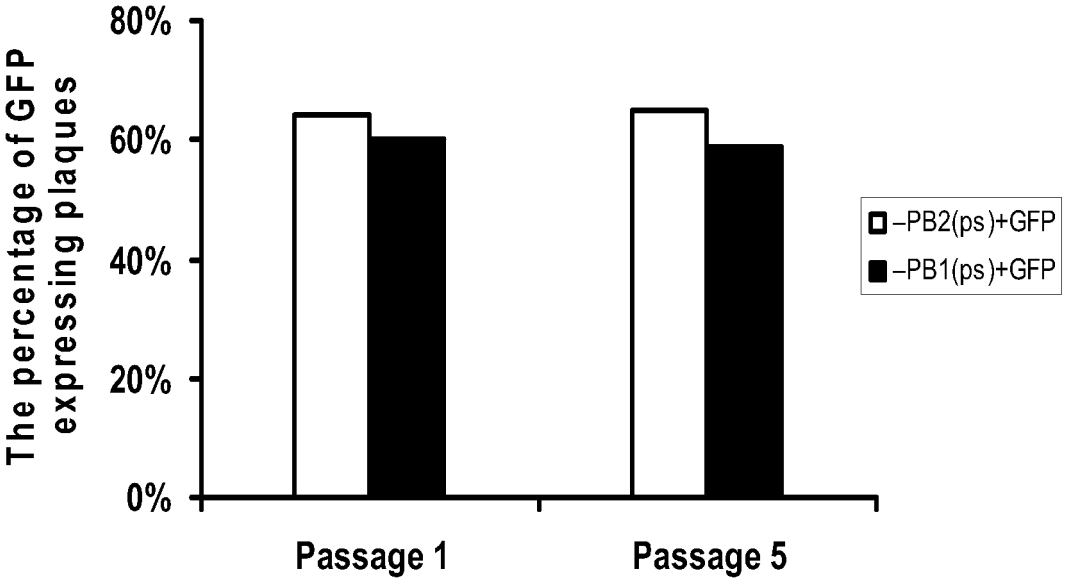

80%
60%
40%
20%
0%
The percentage of GFP expressing plaques
Passage 1
Passage 5
–PB2(ps)+GFP
–PB1(ps)+GFP

Fig. 33

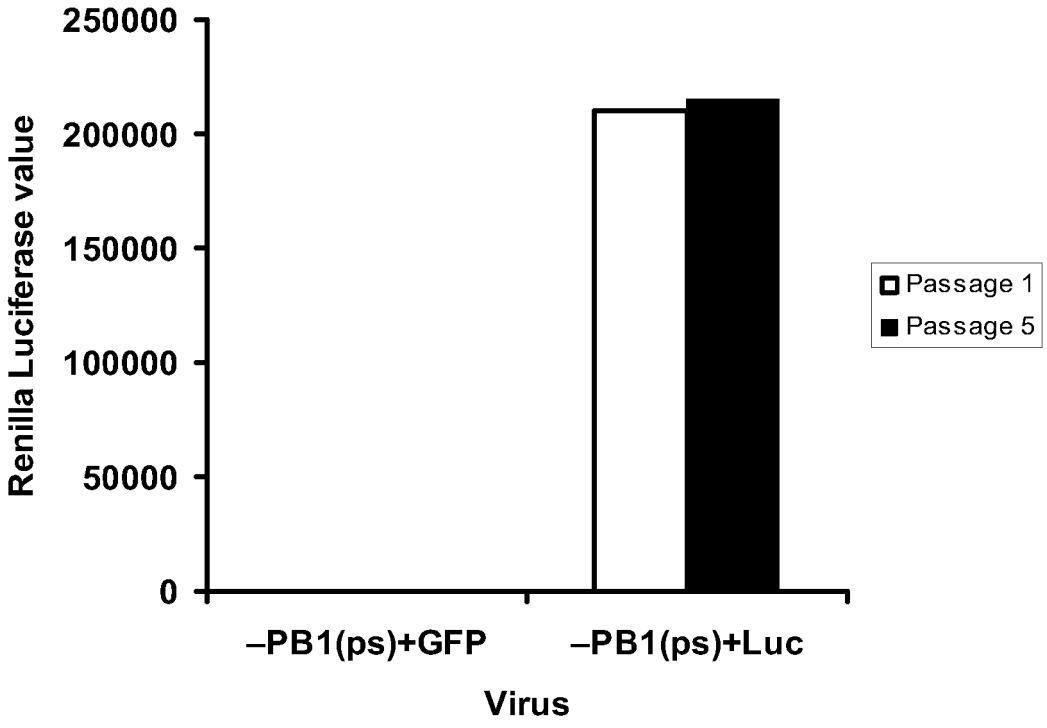

250000
200000
150000
100000
50000
0
Renilla Luciferase value
–PB1(ps)+GFP
–PB1(ps)+Luc
Virus
Passage 1
Passage 5

Fig. 34

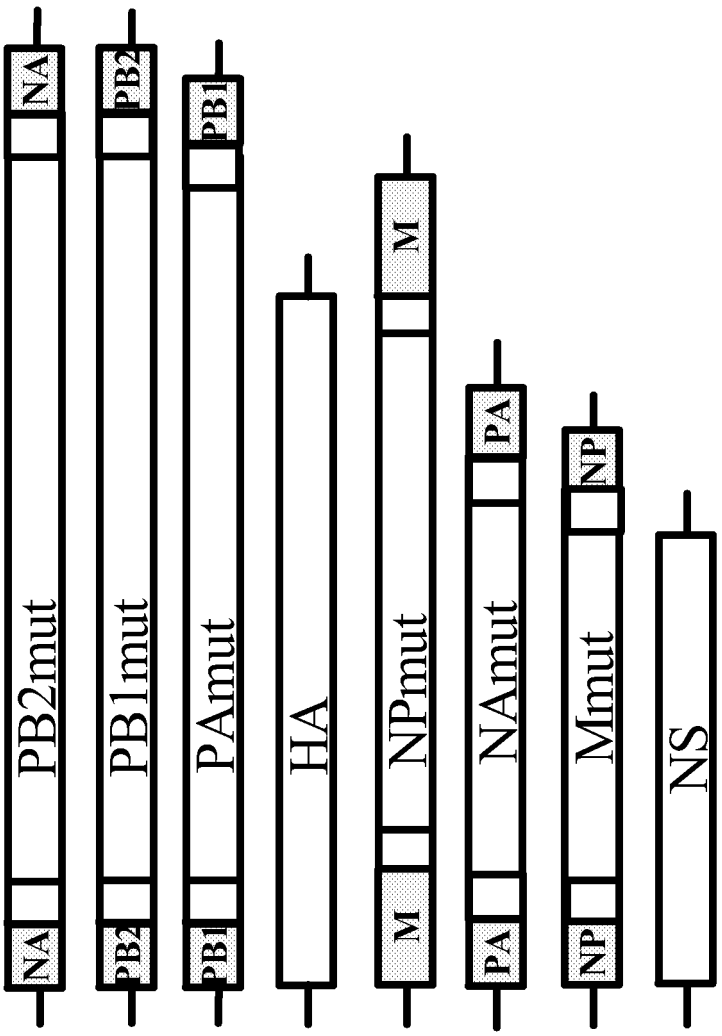

NA-PB2mut-NA
PB2-PB1mut-PB2
PB1-PAmut-PB1
HA
M-NPmut-M
PA-NAmut-PA
NP-Mmut-NP
NS
NA
PB2mut
PB2
PB1mut
PB1
PAmut
HA
M
NPmut
M
PA
NAmut
PA
NP
Mmut
NP
NS

Fig. 35

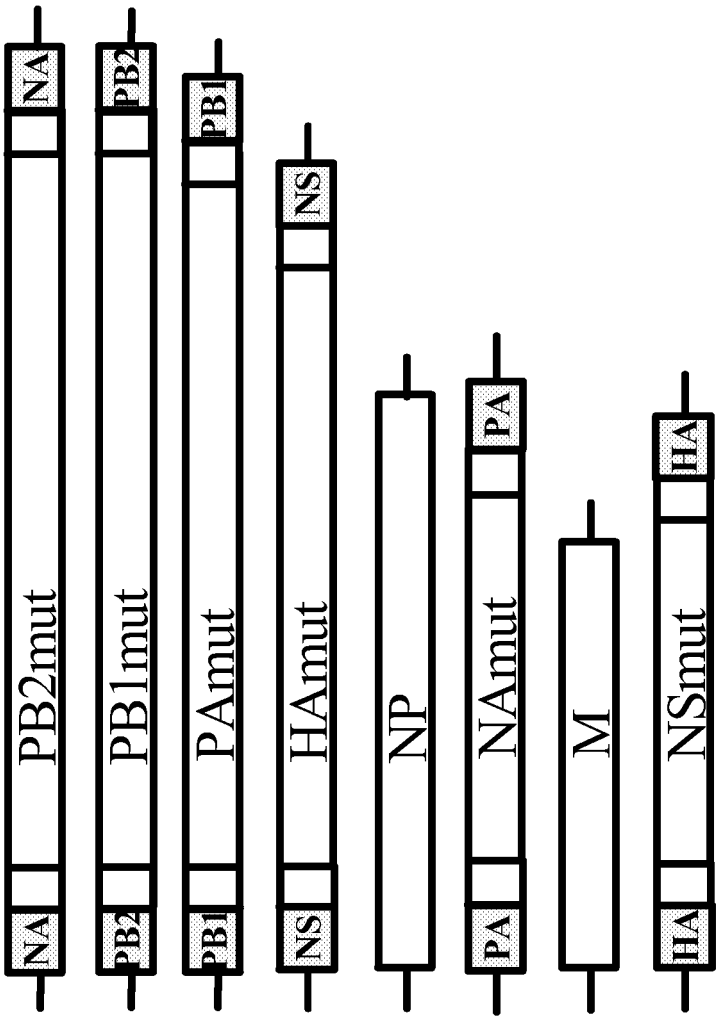

NA-PB2mut-NA
PB2-PB1mut-PB2
PB1-PAmut-PB1
NS-HAmut-NS
NP
PA-NAmut-PA
M
HA-NSmut-HA
NA
PB2mut
PB2
PB1mut
PB1
PAmut
NS
HAmut
NP
PA
NAmut
M
HA
NSmut

Fig. 36

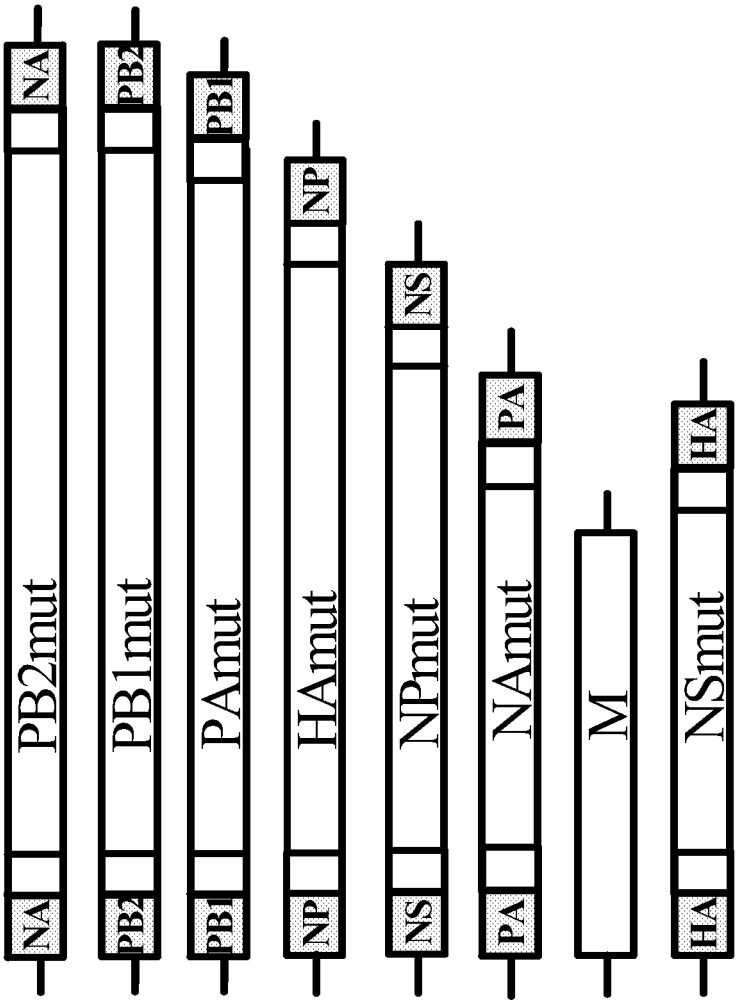

NA-PB2mut-NA
PB2-PB1mut-PB2
PB1-PAmut-PB1
NP-HAmut-NP
NS-NPmut-NS
PA-NAmut-PA
M
HA-NSmut-HA
NA
PB2mut
PB2
PB1mut
PB1
PAmut
NP
HAmut
NS
NPmut
PA
NAmut
HA
NSmut

Fig. 37

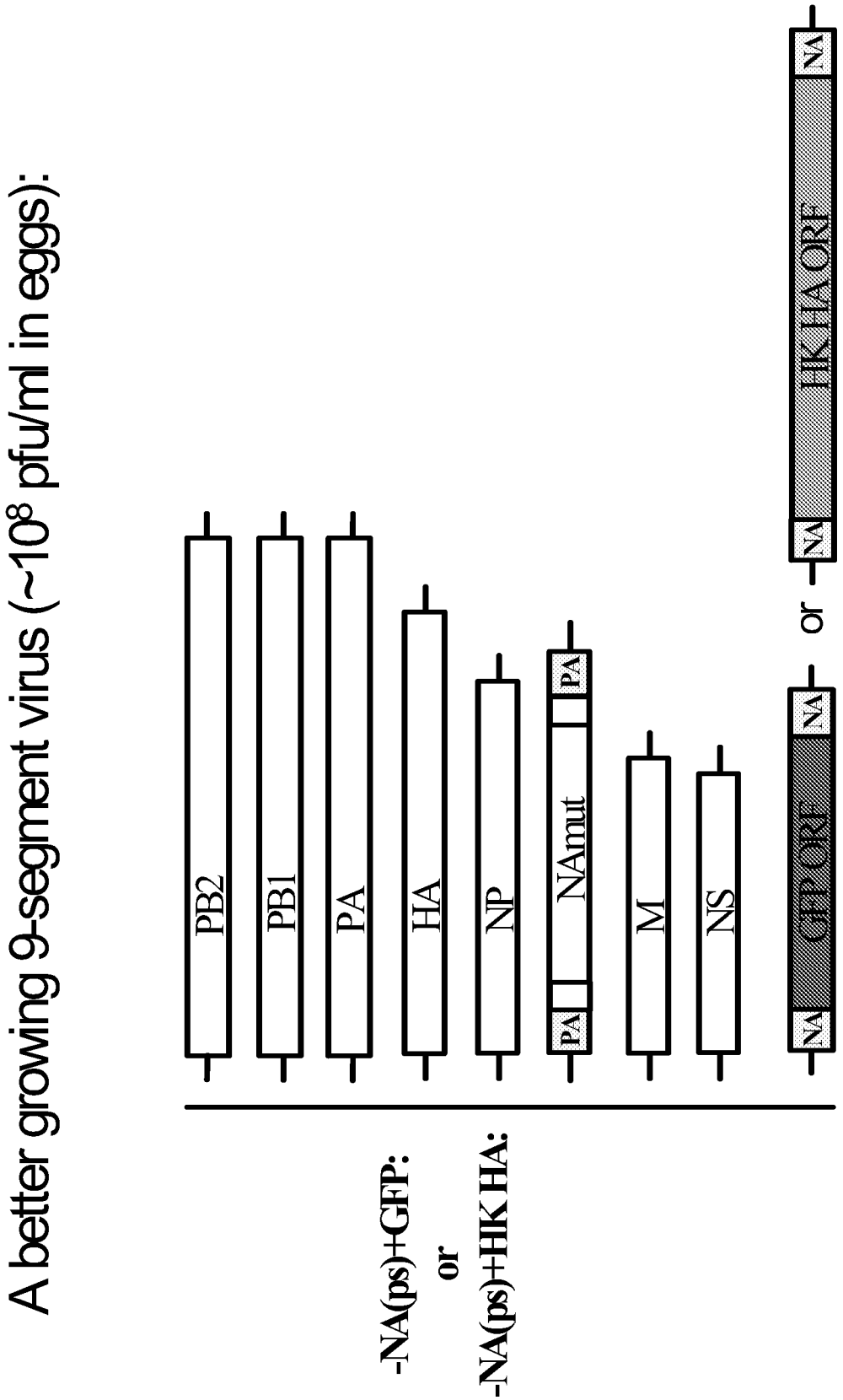

A better growing 9-segment virus (~10^8 pfu/ml in eggs):
-NA(ps)+GFP:
or
-NA(ps)+HK HA:
PB2
PB1
PA
HA
NP
NAmut
PA
M
NS
NA
GFP ORF
NA
or
NA
HK HA ORF
NA

Fig. 38

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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